(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 139 417 B2

(12) **NEW EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the opposition decision:
17.03.1999 Bulletin 1999/11

(45) Mention of the grant of the patent:
26.07.1989 Bulletin 1989/30

(21) Application number: **84305909.8**

(22) Date of filing: **29.08.1984**

(51) Int. Cl.[6]: **A61K 39/245**, A61K 39/00, C12N 15/00, C12N 5/00

(54) Vaccines based on membrane bound proteins and process for making them

Vakzine auf Basis membrangebundener Proteine und Verfahren zu ihrer Herstellung

Vaccins à base de protéines liées à des membranes et procédé pour leur préparation

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

(30) Priority: **30.08.1983 US 527917**
31.10.1983 US 547551
09.03.1984 US 588170

(43) Date of publication of application:
02.05.1985 Bulletin 1985/18

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Description

[0001] This invention relates to membrane bound proteins and derivatives thereof, and to vaccines obtained from them.

Background

[0002] Analysis of the immune response to a variety of infectious agents has been limited by the fact that it has often proved difficult to culture pathogens in quantities sufficient to permit the isolation of important cell surface antigens. The advent of molecular cloning has overcome some of these limitations by providing a means whereby gene products from pathogenic agents can be expressed in virtually unlimited quantities in a non-pathogenic form. Surface antigens from such viruses as influenza (1), foot and mouth disease (2), hepatitis (3), vesicular stomatitis virus (4), rabies (5), and herpes simplex viruses (6) have now been expressed in *E coli* and *S. cerevisiae*, and, in the future, promise to provide improved subunit vaccines. It is clear, however, that the expression of surface antigens in lower organisms is not entirely satisfactory in that potentially significant antigenic determinants may be lost by virtue of imcomplete processing (e.g., proteolysis, glycosylation) or by denaturation during the purification of the cloned gene product.

[0003] This is particularly true in the case of membrane proteins, which, because of hydrophobic transmembrane domains, tend to aggregate and become insoluble when expressed in *E. coli.* Cloned genes coding for membrane proteins can be expressed in mammalian cells where the host cell provides the factors necessary for proper processing, polypeptide folding, and incorporation into the cell membrane (7,8). While these studies show that membrane proteins can be expressed on the surface of a recombinant host cell, and, for example (8), that a truncated membrane protein lacking the hydrophobic carboxy-terminal domain can be slowly secreted from the host cell rather than be bound to it, it is not clear that either the membrane-bound protein thus expressed or the truncated protein thus secreted will be able to act, in fact, to raise antibodies effective against the pathogen from which the protein is derived.

[0004] Herpes Simplex Virus (HSV) is a large DNA virus which occurs in two related, but distinguishable, forms in human infections. At least four of the large number of virus-encoded proteins have been found to be glycosylated and present on the surface of both the virion and the infected cells (9). These glycoproteins, termed gA/B, gC, gD, and gE, are found in both HSV type 1 (HSV1) and HSV type 2 (HSV2), while in the case of HSV 2, an additional glycoprotein (gF) has been reported to be found (10). Although their functions remain somewhat of a mystery, these glycoproteins are undoubtedly involved in virus attachment to cells, cell fusion, and a variety of host immunological responses to virus infection (11). Although HSV 1 and HSV 2 show only ~50 percent DNA sequence homology (12), the glycoproteins appear to be, for the most part, type-common. Thus, gA/B, gD, and gE show a large number of type common antigenic determinants (13—16) while gC, which was previously thought to be completely type-specific (17, 18), has also been found to possess some type-common determinants. Type specific antigenic determinants can, however, be demonstrated using monoclonal antibodies for some of the glycoproteins (10, 19), showing that some amino acid changes have occurred since HSV1 and HSV2 diverged.

[0005] One of the most important glycoproteins with respect to virus neutralization is gD (11). Considerable evidence has been adduced strongly suggesting that the respective gD proteins of HSV-1 and HSV-2 are related. For example, recombination mapping has localized the respective genes to colinear regions in both virus genomes. Amino acid analysis showed gross homology between the two proteins. The gD proteins induce neutralizing antibodies to both type 1 and type 2 viruses in a type-common manner (19—21). In addition, most monoclonal antibodies generated to these glycoproteins are type common, also suggesting a high degree of structural relatedness between the two types of glycoproteins (20). Some monoclonal antibodies, however, were found to react type-specifically, suggesting significant differences between the proteins (19). Peptide maps of the proteins also unambiguously revealed such differences (22a). These results although suggesting that these polypeptides are related, are insufficient to indicate exactly how close the relationship is.

[0006] In order to examine the nature of the type-commonality of HSV-1 and HSV-2 gD proteins, the DNA sequences of the gD genes from HSV1 and HSV2 were determined. The derived amino acid sequences showed similarity. The resultant derived protein sequences were also analyzed for structural differences by using a program designed to determine hydrophobic and hydrophilic regions of the protein. This analysis demonstrated a high degree of conservation on a gross structural level. Although several amino substitutions were found between the two glycoproteins, the vast majority of these substitutions were conservative, suggesting an important structural requirement of this glycoprotein to the virus.

[0007] EP 133063, relevant under Article 54(3) EPC, discloses the expression in bacteria of HSV glycoprotein B lacking C-terminal hydrophobic region, which could alter the conformation of the protein in the E. coli cytoplasm. The document does not deal with expression of the glycoprotein in eukaryotic cells nor suggest the omission of such hydrophobic domain in that situation.

[0008] Nature (1981), 293, pp 620—625, suggests the construction of DNA encoding a hybrid protein comprising a

normally intracellular protein joined to the HA signal sequence, with the suggestion that the resulting protein may be secreted from the host cells.

[0009] According to one aspect of the present invention there is provided a process which comprises producing a truncated, membrane-free derivative of a glycoprotein D of Herpes simplex virus type 1 or type 2, said derivative being devoid of membrane-binding domain whereby the derivative polypeptide is free of said membrane, and having exposed antigenic determinants that raise neutralizing antibodies and provide protection in an immunised subject against in vivo challenge by Herpes simplex virus type 1 and/or type 2, said method comprising expressing DNA encoding said derivative in a stable eukaryotic cell line transfected with said DNA.

[0010] According to another aspect of the present invention there is provided a vaccine comprising a glycosylated truncated, membrane-free derivative of a membrane-bound viral polypeptide, said derivative being the product of expression in, and secretion from, a eukaryotic host cell of recombinant DNA encoding said viral polypeptide but lacking the membrane-binding domain whereby the derivative polypeptide is an immunogenic recombinant secretion product free of said membrane and having exposed antigenic determinants that raise neutralizing antibodies and provide protection in an immunised subject against in vivo challenge by a viral pathogen, the truncated polypeptide being a derivative of a glycoprotein D of a herpes simplex virus type 1 or 2, and the pathogen is herpes simplex virus type 1 and/or type 2.

[0011] In the light of this information about the structure of the gD protein, as described more fully herein, it was decided to express the gD protein DNA in mammalian cells to see whether such was possible, and if possible, whether the expressed protein would bind to the host cell membrane, and whether a truncated form of protein lacking the membrane-binding domain would be secreted from the host cell, and in either of the latter cases whether the expression product proteins could raise antibodies effective against HSV-1 and/or HSV-2. As the results herein demonstrate, these objects have been achieved. In particular, the invention provides using these proteins obtained by recombinant DNA processes as components in a vaccine effective against HSV-1 and HSV-2 viruses. Thus provided are protective vaccines against occurrence of herpes infection and of reduction in frequency and severity of herpes infection recurrence in individuals already infected.

[0012] Glycoproteins obtained by recombinant DNA processes are useful as components in a vaccine against HSV-1 and/or HSV-2 viruses. Specifically, such glycoprotein class includes HSV-1 gC (effective against HSV-1), HSV-2 gF (more properly referred to as an HSV-2 gC), effective against HSV-2, or combinations of the two proteins, effective against both viruses. Other such glycoproteins include gA, gB, and gE. It is believed that a vaccine based upon the combined gC and gD glycoproteins would be significantly more effective as a vaccine than either glycoprotein alone. To further summarize, the present invention involves a vaccine comprising a polypeptide with antigenic determinants capable of specifically raising complementary antibody against HSV-1 and HSV-2 viruses.

[0013] The vaccine comprises a polypeptide with the same antigenic determinants, but which is not functionally associated with the surface membrane. As set out in more detail below, the polypeptide is a truncated, membrane-free derivative of a mebrane-bound polypeptide. The derivative is formed by omission of a membrane-binding domain from the polypeptide, allowing it to be secreted from the recombinant host cell system in which it has been produced.

[0014] As used herein, the term "recombinant" refers to cells which have been transfected with vectors constructed using recombinant DNA technology, and thus transformed with the capability of producing the polypeptide hereof. "Functional assoccation" is meant being bound to the membrane, typically by projecting to both sides of the membrane, in such manner as to expose antigenic determinants folded in a native conformation recognizable by antibody elicited against the native pathogen. "Membrane-bound" in reference to polypeptides hereof refers to a class of polypeptides ordinarily produced in eukaryotic cells and characterized by having a signal sequence which is believed to assist its secretion through various cell membranes as well as a membrane-binding domain (usually hydrophobic in nature and occurring at the C-terminal end) which is thought to preclude its complete secretion through the cell membrane. As such, it remains functionally associated or bound to the membrane.

[0015] In the present invention, membrane-free preparations may be obtained by creation of a secretion system. As described in more detail below, such secreted polypeptide possesses at least some of the antigenic sites necessary for antibody stimulation.

[0016] In the accompanying drawings:

Figures 1A and 1B show the DNA and deduced amino acid sequences of the HSV-1 and HSV-2 gD genes and surrounding flanking regions;

Figure 2 shows a hydropathy analysis of the gD proteins from HSV-1 and HSV-2 proteins;

Figure 3 is a diagram of the plasmid pgD-*dhfr*, constructed for the expression of a membrane-bound form of HSV-1 glycoprotein D;

Figure 4 shows the result of labelling of gD12 cells with human antibodies against HSV, (A) being a visualiation with phase contrast optics, (B) a fluorescence image of the same cells;

Figure 5 shows radioimmunprecipitations of cloned gD from the gD12 cell line hereof and native gD from HSV-1

infected human cells;

Figure 6 shows the binding of human anti-HSV antibodies to gD12 cells and the parental CHO cell line.

Figure 7 is a schematic representation of HSV-1 gD protein and illustrates the locations of signal sequence and membrane-binding domain.

Figure 8 is a diagram of the expression plasmid pgDtrunc-dhfr for a secreted form of HSV-1 gD protein.

Figure 9 shows radioimmunoprecipitations from the gD10.2 cell line hereof.

Figure 10 shows radioimmunoprecipitations from preamplified and amplified gD10.2 cell lines.

Figure 11 demonstrates the degree of amplification achieved with the Mtx amplified gD10.2 cell line.

Detailed description (Examples)

Example 1

Example 1 relates to gD protein.

Virus growth and viral DNA isolation

[0017] HSV1 (strain Hzt) and HSV2 (strain G) were grown on Hep 2 cells at 37° and at 33°C, respectively. The viral DNA was isolated from infected cell cultures by proteinase K digestion and CsCl banding (23).

Cloning of the gD genes of HSV1 and HSV2

[0018] Previous mapping and cloning studies had localized the HSV1 gD gene to a ~6.6 kb BamHI fragment (6,24). HSV1 DNA was cleaved with BamHI, and the 6—7 kb region was isolated by agarose gel electrophoresis. This fragment was ligated into BamHI-digested pBR322, and the resultant mixture was used to transform *E. coli* strain 294 (ATCC No. 31446). The ampicillin resistant, tetracycline sensitive plasmids were screened for the proper HSV1 fragment by restriction enzyme digestion. The correct gD containing Sst1 fragment was subcloned into Sst1-digested plasmid pFM3 (European Patent Application Publication No. 0068693; 5 January 1983).

[0019] Although the gD gene from HSV2 was previously mapped by recombination with HSV1, the exact location of this gene was unknown. Therefore, an ~10 kb HindIII fragment from the small unique region of the HSV2 genome (4) was ligated into the HindIII site of the bacteriophage lambda cloning vector 590 (25). *In vitro* packaged phage were plated at low density and screened by the Benton-Davis procedure with a ^{32}P-labeled subclone of the gD gene from HSV1 (26). Positively hybridizing plaques were grown, the DNA isolated, and the gD gene localized by Southern blotting and hybridization with the ^{32}P-labelled HSV1 gD gene (27). The positively hybridizing, HSV2 gD containing fragments were subcloned into the plasmid pUC9 (28).

DNA Sequence determination and computer analysis

[0020] Various fragments from the HSV1 and HSV2 gD genes were subcloned into the m13 phage vector mp9 (29), and were sequenced by the dideoxynucleotide method of Sanger (30).

[0021] The nucleotide sequences were analyzed using the HOM program (31). The hydropathy of the deduced protein sequence was analyzed using a width of 12 and a jump of 1 (31a).

Cloning of the gD regions from HSV1 and HSV2

[0022] Other studies had localized the HSV1 gD gene to the 6.6 kb *Bam*HI J fragment according to the nomenclature of Roizman (6, 12, 24). Isolation and sequencing of part of this fragment showed that this fragment contained the HSV1 gD gene. Since one might expect that the DNA sequences of the HSV1 gD gene would be relatively homologous to the HSV2 gD gene, this fragment was used as a probe for the isolation of the gD gene from the HSV2 genome.

[0023] Since most of the genes from the HSV1 and HSV2 genomes appear to map colinearly (35), the region from the small unique region of the HSV2 genome which corresponded to the HSV1 gD region (the HindIII L fragment (12)), was cloned into a lambda phage vector. Screening of the resultant plaques with a ^{32}P-labelled HSV1 gD gene subclone revealed positively hybridizing plaques, suggesting that there was indeed nucleic acid sequence homology between the two virus genomes in this region. Isolation of the phage DNA and subsequent Southern blot analysis revealed the region of this fragment which corresponded to the gD gene. This region was subcloned for DNA sequence analysis.

The coding regions

[0024] Figure 1 illustrates the two gD DNA sequences compared with the HOM program (31). Nucleotide number 1 is chosen as the A of the ATG initiator methionine. Gaps have been introduced by the HOM computer program to maximize the sequence homologies (31). Nucleotide differences are shown by the symbol (*), while amino acid differences are shown boxed. Amino acid differences between the HSV1 gD sequence reported here, determined for the Hzt strain of HSV1, and that reported by Watson *et al* (6) for the Patton strain, are depicted by the symbol (+). The start of HSV1 gD gene transcription, shown by an arrow, is from Watson *et al* (32). Possible N-linked glycosylation sites are shown shaded. Two possble "TATA" sequences are shown 5, to the start of gD transcription, while a third possible "TATA" sequence is shown 5, to a second open reading frame at the 3' end of the HSV2 sequence. Two regions of non-coding sequence homology should be noted 5' to the gD genes and 5' to the second open reading frame from the HSV2 sequence.

The hydropathy of gD proteins

[0025] The hydropathy of each glycoprotein was analyzed using the program developed by Hopp *et al* (31a). As shown in Figure 2, a hydrophobic transmembrane domain exists at the 3'-end of the gene. Twelve amino acid long stretches were analyzed, and the average hydropathy was calculated. Residue differences between the two glycoproteins are shown, with conservative changes marked (*) and non-conservative changes marked (+). A) HSV1 gD protein hydropathy, B) HSV2 gD protein hydropathy.

[0026] The DNA sequence analysis demonstrates that the HSV1 and HSV2 gD proteins are 80 percent homologous. The majority of the differences found between these two proteins were in the amino and carboxy terminal regions. The amino-terminal region of these proteins contains a highly hydrophobic region which contains an arginine residue near the amino terminal methionine. This hydrophobic domain is the signal sequence which is characteristic of secreted and membrane-bound proteins and which presumably functions to direct at least a portion of the protein into the lumen of the endoplasmic reticulum (33). A comparison of the first twenty amino-terminal amino acids showed that there were a total of 12 differences between the type 1 and type 2 genes. Virtually all of the differences, however, are conservative since they encode other hydrophobic amino acids. The exceptions are the gly-arg replacement at residue 3 and the arg-gly replacement at residue 7. Although these replacements are not conservative, they do not change the net structure of the signal domain. Both genes maintain a positively charged residue within the first 10 amino acids.

[0027] The hydropathy plot in Figure 2 revealed a hydrophilic carboxy-terminal domain preceded by a hydrophobic region. This structure is characteristic of membrane-bound glycoproteins and has been previously found in other viral surface antigens (5, 34). Its function is to anchor the protein in the cellular and viral membranes and, as such, performs an important role for virus infection. Twelve amino acid changes in this region of the gD proteins from residues 333 to 362 were found, most of which are conservative. This suggests that the only criterion for the amino acids in this region is that they be predominantly apolar in order to span the lipid bilayer. In addition, the region after the membrane domain (residues 363—375), which probably serves to anchor the protein in the membrane (33), shows 5 changes in its first 13 residues followed by a long homologous stretch. This result suggests that the initial 10—15 residues in the carboxy-terminal hydrophilic domain may only serve an anchoring function and therefore only need to be charged, while the supernatant 23 residues may serve some other function important to the gD protein specifically.

[0028] Although many other amino acid changes are found throughout these two proteins, the vast majority of the changes are conservative. This fact is underlined by the structure revealed by the hydropathy program shown in Figure 2. As can be seen in this comparison, the two glycoproteins show very similar plots. The amino acid changes which are not conservative do not appear to change the hydropathy of the protein.

Expression of the HSV-1 gD

[0029] In order to establish a permanent membrane-bound gD producing cell line, the gD containing fragment was ligated (Figure 3) into a mammalian expression vector (36) containing the selectable marker, dihydrofolate reductase (*dhfr*). Figure 3 shows a diagram of the plasmid, pgD-*dhfr*, constructed for the expression of HSV-1 glycoprotein D. The expression plasmid consisted of the origin of replication and the β-lactamase gene (amp^r) derived from the *E. coli* plasmid pBR322 (37), a cDNA insert encoding mouse *dhfr* (36, 38) under control of the SV-40 early promoter and a 4.6 kb *Hind*III to *Bam*HI fragment containing the gD gene also under control of the SV-40 early promoter. The *Hind*III end of this fragment lies 74 bp to the 5' side of the initiator methionine codon and includes the mRNA cap site. The *Hind*III site lies 250 bp to the 3' side of the Goldberg-Hogness box of the SV-40 promoter. The coding region of the gD-containing fragment is 1179 bp long and adjoins a large (1.9 kb) 3' region which contains at least part of the glycoprotein E gene (24, 32), a translational stop codon, and a polyadenylation site.

[0030] The plasmid pgD.dhfr was constructed as follows: The 4.6 kilobase HindIII-Bam H1 fragment containing the

entire gD coding sequence was isolated from the Bam H1 fragment cloned from the HSV 1 genome (see above). The 2.8 kilobase HindIII-Sal 1 fragment containing an SV40 origin-early promoter and the pBR322 ampicillin resistance gene and origin of DNA replication were isolated from the plasmid pEHBal 14. The 2.1 kilobase Sal 1-Bam H1 fragment containing a murine dihydrofolate reductase cDNA clone under the control of a second SV40 origin-early promoter was isolated from the plasmid pE348HBV E400D22 (36). These three fragments were ligated together in a triple ligation using T4 DNA ligase, and the resultant mixture was used to transform *E. coli* strain 294. The resultant colonies were grown and the plasmid DNA screened by digestion with Sac 2. The correct DNA construction pgD.dhfr (Figure 3) was used for further transfection studies.

[0031] The plasmid was introduced into Chinese Hamster Ovary cells (CHO) deficient in the production of *dhfr* (39) using a calcium phosphate precipitation method (40). Colonies capable of growth in media lacking hypoxanthine, glycine, and thymidine were obtained and nine *dhfr*$^+$ clones were analyzed. Of these, gD could be detected in five colonies using anti-HSV-1 antibodies in radioimmunoprecipitation and indirect immunofluorescence assays. One of the five lines (gD12) was designated for further study. In order to characterize the cloned gD gene product, gD12 cells were metabolically labeled with ^{35}S-methionine or ^{3}H-glucosamine and analyzedd by radioimmunoprecipitation. The procedure used was as follows: Cells were grown in Ham's F12 medium (Gibco) supplemented with 7 percent commercially dialyzed fetal bovine serum (Gibco), penicillin (100 u/ml), and streptomycin (100 u/ml). When the cultures were approximately 80 percent confluent, the medium was removed, the cells were washed twice with phosphate buffered saline (PBS), and labeling medium (Dulbecco's modified Eagle's medium containing either one-tenth the normal concentration of methionine or glucose) was added to a final concentration of 0.064 ml/cm^2. Either ^{35}S-methionine (SJ.204, Amersham Int.) (50—75 μCi/ml) or ^{3}H-glucosamine (100 μCi/ml) was added and the cells were grown for an additional 18—20 hr. After labeling, the medium was harvested and the cells were washed twice in PBS, and removed from the culture dish by treatment with PBS containing 0.02 percent EDTA. The cells were then solubilized in lysis buffer consisting of: PBS, 3 percent NP-40, 0.1 percent bovine serum albumin, 5×10^{-5} M phenylmethylsulfonyl fluoride, and 0.017 TIU/ml of apoprotinin and the resultant lysate was clarified by centrifugation at 12,000×g. For immunoprecipitation reactions cell lysates were diluted 3-fold with PBS and aliquots (typically 180 μl) were mixed with 2—5 μl of antisera and incubated at 4°C for 30 min. Immune complexes were then absorbed to fixed S. aureus cells by the method of Kessler (40a) and were precipitated by centrifugation at 12,000×g for 305. The S. aureus cells were then washed 3 times with wash buffer (PBS, 1 percent NP-40, 0.3 percent sodium dodecyl sulfate), and the immune complexes were eluted with 20 μl of polyacrylamide gel sample buffer (62.5 mM Tris-HCl buffer, pH 6.8 containing 10 percent glycerol, 5 percent 2-mercaptoethanol, 0.01 percent bromophenol blue) at 90°C for 3 min. After centrifugation for 30 s the supernatants were applied to 10 percent polyacrylamide slab gels according to the method of Laemmli (45).

[0032] Figure 5A compares autoradiographs obtained with the gD12 cell line and HSV-1 infected cells: control immunoprecipitation from the gD12 cell lysate with normal rabbit serum (lane 1); immunoprecipitation of native gD grown in HEL cells (lane 2) and A549 cells (lane 3) with the monoclonal anti-gD antibody, 55-S (41); immunoprecipitation of cloned gD from the gD12 cell lysate with polyclonal rabbit antibodies (Dako Corp.) to HSV-1 (lane 4), and the monoclonal antibody, 55-S (lane 5); immunoprecipitation of cloned gD from the gD12 cells metabolically labeled with ^{3}H-glucosamine with polyclonal rabbit anti-HSV-1 antibodies (lane 6).

[0033] It is seen (lanes 4 and 5) that a diffuse band of 59—60 kd was specifically precipitated from the gD12 cell line using either rabbit anti-HSV-1 antibodies or the monoclonal anti-gD antibody, 55-S, specific for the HSV-1 protein (41). This molecular weight agrees well with that reported for gD isolated from HSV-1 infected KB cells (42). It is seen that the same monoclonal antibody precipitated proteins of similar but different molecular weights from HSV-1 infected human cell lines. The major product precipitated from the A549 human lung carcinoma cell line (lane 2) was 53 kd and that precipitated from the human embryonic lung cell line (HEL) was 56 kd (lane 3). Previous studies (43) have shown that the molecular weight of HSV glycoproteins varies depending on the host cell and that these differences are due to differences in glycosylation. To determine whether the gD protein produced in CHO cells was, in fact, glycosylated, the cells were metabolically labeled with ^{3}H-glucosamine. Because bands of identical molecular weights (lanes 5 and 6) were precipitated after metabolic labeling with ^{35}S-methionine or ^{3}H-glucosamine, we concluded that the gD protein produced in CHO cells is glycosylated.

[0034] The human cell lines A549 (ATCC CCL 185) and HEL 299 (ATCC CCL 137) were grown to confluence in 3.5 cm tissue culture dishes and infected with HSV-1 at multiplicity of 10 pfu per cell. Virus infected cells were labeled by a method similar to that described by Cohen *et al.* (44). 4 hr after infection the medium was removed and the cells were washed once with fresh medium (Dulbecco's modified Eagle's medium and once with phosphate-buffered saline (PBS). Fresh medium containing one-tenth the normal concentration of methionine was then added to the cells along with ^{35}S-methionine (Amersham International) to a final concentration of 75 μCi per ml of medium. The cells were grown an additional 20 hr and then harvested by treatment of washed cells with PBS containing EDTA (0.02 percent). Viral proteins were solubilized in lysis buffer consisting of PBS, 3 percent NP-40, 1 percent bovine serum albumin, 5×10^{-5}M phenylmethylsulfonyl fluoride, and 0.017 TIU/ml of apoprotinin. The resultant lysate was clarified by centrifugation at 12,000×g in a microcentrifuge. For immunoprecipitation reactions the cell or virus lysates were diluted 3-fold with phosphate buff-

ered saline, mixed with 2—5 µl of the appropriate antiserum and incubated for 30 min at 4°C. Antibody-antigen complexes were removed from the reaction medium by the addition of 25 µl of a 10 percent solution fixed *S. aureus* (Kessler (40a)) and were precipitated by centrifugation at 12,000×g for 30 s. The *S. aureus* cells were then washed 3 times with wash buffer (PBS, 1 percent NP-40, 0.3 percent sodium dodecyl sulfate), and the cells suspended in 20 µl of polyacrylamide gel sample buffer (10 percent glycerol, 5 percent 2-mercaptoethanol, 0.0625 M in pH 6.8 Tris buffer, 0.01 percent bromophenol blue) and incubated at 90°C for 3 min. After centrifugation (12,000×g) for 30 s the supernatants were applied to 10 percent polyacrylamide slab gels (45).

[0035] To further explore the post-translational processing of cloned gD, pulse-chase studies were conducted. Figure 5B shows immunoprecipitation of cloned gD from gD-12 cells with rabbit anti-HSV-1 antibodies (Dako, Corp.) at various times after pulse labeling with ^{35}S-methionine. Figure 5B shows a pulse labeling of the gD12 cells. In these studies, cells were grown to confluence in 10 cm tissue culture dishes and labeled with ^{35}S-methionine as described above with the exception that the labeling reaction was carried out for 15 min. on ice, the cells washed 3 times with fresh medium, and then returned to the incubator and incubated at 37°C for various times. The reactions were terminated by washing the cells in cold phosphate-buffered saline and solubilizing the cells as described above. Proteins were immunoprecipitated at the following times after pulse labeling: lane 1, 5 min; lane 2, 15 min; lane 3, 30 min; lane 4, 60 min; lane 5, 120 min. The precursor form of gD with a molecular weight of 51 kd was specifically precipitated from the gD12 cell line 5 min after a pulse with ^{35}S-methionine, and this precursor chased into the higher molecular weight form (59 kd) after approximately 60 min. From these studies we estimate the half-time for this post-translational event to be approximately 45 min. The precursor-product relationship between the 51 kd band and 59 kd band closely resembles that reported for virus produced gD (14, 42, 46, 47) and the kinetics of this process are similar to those described by Cohen *et al* (42). In virus infected cells the difference in molecular weights between the precursor and the product has been attributed to both N-linked and O-linked oligosaccharides (48).

[0036] To determine whether gD was exported to the cell surface, indirect immunofluorescence studies were conducted. In these studies rabbit, mouse, and human anti-HSV antibodies were reacted with unfixed cells under conditions which do not permiablize the cell membrane (49). gD12 cells and the parental CHO cells (1:1) ratio) were plated onto glass coverslips (2.2×2.2 cm) and grown until the cells were approximately 60 percent confluent. Human serum known to contain antibodies to HSV-1 (50) was diluted forty-fold with phosphate buffered saline (PBS) and 100 µl was pipetted onto washed cells and was incubated for 30 min at room temperature in a humdified chamber. The cells were immersed 3 times in PBS to wash away unbound antibody and then were incubated with 100 µl of 20-fold diluted tetramethylrhodamine isothiocyanate-labeled goat anti-human IgG antibodies (Cappel Laboratories) for an additional 30 min. The unbound labelled antibody was washed away with PBS and the cells were dehydrated in ice cold 50 percent ethanol and 100 percent ethanol and rehydrated with glycerol on a microscope slide (49). The cells were then viewed under phase-contrast and fluorescence optics in fluorescence microscope (Zeiss). Figure 4 shows: A, gD12 and CHO cells viewed visualized with phase contrast optics; B, fluorescence image of the same cells as in A. Comparison of the phase-contrast images with the fluorescence images showed that the gD12 cells were heavily labeled, while the parental CHO cells bound little or no labelled antibody. In control experiments with normal mouse sera, normal rabbit sera, or human sera known to be negative for HSV antibodies, no specific labeling of the cells could be detected. These studies suggested that the gD was exported to the cell surface. Experiments with CHO and gD12 cells fixed prior to labelling with agents known to permiablize the cell membrane (methanol or acetone) gave a different labeling pattern. In these studies we observed heavy perinuclear labeling of the gD12 cells with anti-HSV-1 antibodies, and no specific labelling of the CHO cells.

[0037] In order to detrmine whether gD12 cells expressed antigenic determinants relevant to human HSV-1 and HSV-2 infections, the binding of antibodies from individuals known to possess anti-HSV-1 or anti-HSV-2 antibodies (50) was examined. Radioimmunoprecipitation of lysates from metabolically labelled gD12 cells gave results comparable to those obtained with rodent anti-HSV sera (Figure 5). Similarly, human anti-HSV-1 sera gave specific labeling of gD12 cells in an indirect immunofluorescence assay (Figure 4) and did not label the parental CHO cell line. Taken together, the results obtained with various rodent anti-HSV-1 and HSV-2 antisera, monoclonal anti-gD antibodies and human anti-HSV antisera provide evidence that gD expressed on the surface of gD12 cells possesses a number of antigenic determinants in common with the native virus and that the structure of these determinants is not dependent on interactions with other HSV-1 proteins. The fact that one of the monoclonal antibodies tested (1-S) is known to neutralize HSV-1 *in vitro* (41) and *in vivo* (51) demonstrates that the gD produced in CHO cells possesses at least one of the neutralizing antigenic determinants in common with the native virus.

[0038] In order to have a quantitative measure of the binding of anti-HSV antibodies to gD12 cells, an enzyme-linked immunosorbtion assay (ELISA) was developed (52). In these studies gD12 cells and CHO cells were plated and chemically fixed into alternate wells of 96 well microtiter tissue culture plates. Various antisera known to possess antibodies to HSV were then serially diluted and allowed to react with the fixed cells. At the end of the assay, the absorbance in each well was measured and normal binding curves were constructed. The specific binding of antibodies to the gD12 cells was determined by subtracting the values obtained with the parental CHO cells from those obtained from the gD12

cells. Specific binding by high titer sera could be detected at dilutions of 1:10,000.

[0039] We compared serum titers determined using the gD12 cell ELISA assay with anti-HSV-1 and anti-HSV-2 titers determined by conventional methods. Human sera previously titered (50) against HSV by conventional assays, i.e., inhibition of hemagglutination (IHA) or complement fixation (CF) was serially diluted into wells of microtiter plates containing either gD12 cells or the parental CHO cell line and the binding of anti-gD antibodies was monitored in an ELISA assay; gD12 cells and the parental CHO cells were seeded into alternate wells of 96 well microtiter tissue culture plates (Falcon Labware) and were grown to confluence in F12 medium (GIBCO) containing 10 percent fetal bovine serum. The cells were washed three times with phosphate-buffered saline (PBS) and then were chemically fixed with 0.0625 percent glutaraldehyde in PBS. The cells were again washed three times with PBS and stored until needed at 4° in PBS containing 1 percent bovine serum albumin, 100 mM glycine 1 mM NaN_3. To measure anti-gD antibody titers, the cells were washed with PBS, and serially diluted antisera was allowed to react with the fixed cells (50 µl final volume) for 1 hr at room temperature. Unbound antibody was washed away and the cells were incubated with 50 µl of 1:2000 diluted goat anti-human IgG coupled to horseradish peroxidase (Tago, Inc.). The enzyme-linked antibody was allowed to react for one hour at room temperature, and the cells were then washed three times with PBS. After incubation, the peroxidase substrate, o-phenylene diamine, was added (200 µl) and the reaction was allowed to proceed for 10 min. The reaction was terminated by the addition of 2.5 M H_2SO_4 (50 µl) and the absorbance of the reaction medium from each well was determined with an automated plate-reading spectrophotometer (Titertek). In Figure 6, the serum represented by the open and closed circles exhibited a HSV-1 CF titer of 128 and HSV-1 and HSV-2 IHA titers of 4096. The serum represented by open and closed squares exhibited a HSV-1 CF titer of <8 and HSV-1 and HSV-2 IHA titers of <8. A closed circle and closed square indicates binding to gD12 cells; open circle and open square indicates binding to CHO cells. B, closed circle and closed square represents the specific binding to gD12 cells calculated by subtraction of the values in A. In Figure 6 it can be seen that a serum with a high anti-HSV titer determined by conventional assays gave a high ELISA titer, while another serum with low anti-HSV titers gave no detectable binding in the gD12 ELISA.

[0040] The studies described demonstrate that stable cell lines constitutively express on their surface a transfected gene product which binds with antibodies generated by herpes virus infection.

Immunization of mice with gD12 cells

[0041] Twenty female BALB/c mice (5 weeks of age) were obtained from Simonsen Laboratories (Gilroy, California). The mice were divided into two groups of 10 mice each: an "experimental" group and a "control" group. Each mouse in the experimental group was injected with gD12 cells known to express HSV-1 glycoprotein D on their surface. Each mouse in the control group was injected with the parental Chinese hampster ovary cell line (CHO cells) from which the gD12 cell line was derived. For immunization of mice both types of cells were grown to confluence in 15 cm tissue culture dishes. The CHO cells were grown in Hams F12 medium (GIBCO) supplemented with 7 percent commercially dialyzed fetal bovine serum (GIBCO), penicillin (100 U/ml), and streptomycin (100 U/ml). The gD12 cells were grown in the same medium lacking glycine, hypoxanthine, and thymidine. To harvest the cells, each dish was washed twice with 15 ml of phosphate buffered saline (PBS) and then treated with 15 ml of PBS containing 0.02 percent EDTA. After 15—20 min. the cells were then removed from the dish and pelleted by centrifugation for 5 min. at full speed in a clinical centrifuge (IEC model CL clinical centrifuge, rotor model 221). The supernatant was discarded and the cells were resuspended in PBS to a final concentration of 1 ml PBS per each 15 cm dish of cells. Each mouse was then injected with 0.5 ml of cell suspension (~5×10^6 cells) distributed as follows: 0.25 ml injected interperitoneally, and 0.25 ml injected subcutaneously in the loose skin of the back of the neck. The mice were then boosted twice with fresh cells (prepared as described above) on day 38 and day 55 after the initial immunization. Mice were bled via the tail vein on day 68 to obtain seri for in vitro neutralization studies. Mice were challenged with HSV-1 (MacIntyre strain) on day 70. The virus challenge entailed an interperitoneal injection of 2×10^7 pfu of virus into each mouse. The mice were scored daily for mortality and every other day for weight change and the onset of paralysis. All of the mice in the control group died within 7 days of the virus challenge, while all of the experimental mice were protected and showed no sign of infection. These studies conclude that immunization with the gD12 cells protect from a lethal HSV-1 virus challenge.

[0042] A variety of transfection schemes are possible, of course, using a variety of selectable markers. For example, mouse L cells can be usefully transfected using a mutant *dhfr* gene as a selectable marker. The gD gene was transfected into such cells via a vector harboring such a marker. In principle, the strategy which we have described could be applied to any situation where the expression of a membrane protein is desired.

Expression of a truncated form of the gD gene

[0043] The foregoing description relates to the production of membrane-bound gD protein. However, as discussed above in relation to Figure 2, analysis of the amino acid sequences of the gD protein of HSV-1 and HSV-2 identified in each case a hydrophobic/hydrophilic carboxy-terminal membrane binding domain (Figure 7).

A schematic diagram of the HSV 1 glycoprotein D (gD)

[0044] Hydrophobic (shaded) and hydrophilic (market+) regions of the protein were determined from the hydropathy analysis (31a) of the gD protein sequence derived from the gene sequence. Only those regions thought to be important for membrane localization and binding are shown. The functional domains are: a) the signal sequence (33), b) the hydrophobic transmembrane domain, and c) the charged membrane anchor. The three putative N-linked glycosylation sites are shown by the letter G. The expression plasmid consisted of the pBR322 bacterial origin of replication and ampicillin resistance gene, a cDNA insert encoding the murine dihydrofolate reductase gene under the transcriptional control of the SV40 early promoter (53) and a HindIII-Hinf1 fragment which encodes the first 300 amino acids of gD under the transcriptional control of a second SV40 early promoter. The HindIII site of this fragment lies 74 bp to the 5' side of the initiator methionine of the gD gene. The HindIII site of the SV-40 early region vector (36) lies 250 bp to the 3' side of the Goldberg-Hogness box of the SV40 promoter. The Hinf1 site (blunted with Klenow DNA polymerase and 4 deoxynucleotide triphosphates) is ligated to the Hpa1 site of the 3' nontranslated region of the hepatitis B virus surface antigen gene (36). This method is also useful for preparing a truncated HSV-2 gene. The resultant sequence creates a stop codon (TAA) immediately after amino acid 300 of the gD gene. The transcription termination and polyadenylation sites for the truncated gD gene transcript are encoded by the 3' untranslated region of the hepatitis B surface antigen gene (36).

[0045] The plasmid pgDtrunc.dhfr was constructed as follows: The 2.9 kilobase gD-containing Sac 1 fragment was isolated from the Bam H1 fragment cloned from the HSV 1 genome (see above) in the plasmid pFM3 (see above) cut with Sac 1. A 1.6 kilobase HindIII-Bst N1 fragment containing the entire gD gene was subcloned into HindIII-Bst N1 digested pFM42 (EPO Application No. 68693). This plasmid was then cut with Hinf 1, blunted with Klenow DNA polymerase and four deoxynucleotide triphosphates, and then subsequently cut with HindIII. The 960 base pair HindIII-blunt Hinf 1 fragment containing the truncated gD gene was isolated and ligated to HindIII-Hpa1 digested pEHBal14. The resultant construction (pgDCos-Trunc) contained the truncated gD gene with the hepatitis B surface antigen gene at its 3 prime end. A 2.3 kilobase HindIII-Bam H1 fragment containing the truncated gD gene was isolated from pgD-Cos-trunc. The 2.8 kilobase fragment containing the SV 40 origin-early promoter and the pBR322 ampicillin resistance gene and bacterial origin of replication were isolated from the plasmid pEHBal 14. The 2.1 kilobase fragment containing the murine dihydrofolate reductase cDNA clone under the transcriptional control of a second SV 40 early promoter was isolated from the plasmid pE348HBVE400D22 (36). These three fragments were ligated together with T4 DNA ligase, and the resultant mixture was used to transform *E. coli* strain 294. Plasmid DNA from the resultant colonies were screened with Sac 2, and the correct construction pgDtrunc.dhfr (Figure 8) was used for further transfection studies.

[0046] Plasmid pEHBal 14 was constructed by cleaving pE342ΔR1 (described below), an SV40-hepatitis chimera, with Xbal, which cleaves once in the coding region of the HBV surface antigen, and sequentially removing sequences surrounding this Xba I site by using nuclease Bal31. The plasmid was ligated in the presence of the synthetic oligonucleotide 5'-AGCTGAATTC, which joins the HBV DNA with a HindIII restriction site.

[0047] Resulting plasmids were screened for an Eco R1-Hind III fragment of ~150 b.p. pEHBal 14 was sequenced, which verified that a HindIII site had been placed at a point just upstream of where the HBsAg initiation codon is normally found. This construction thus places a unique HindIII site suitable for cloning at a position where a highly expressed protein (HBsAg) initiates translation. Any putative signals necessary for high expression of a protein should be present on this 5' leader sequence.

[0048] Plasmid pE342 which expresses HBV surface antigen (also referred to as pHBs348-E) has been described by Levinson et al, EPO Publication No.0073656 March 9, 1983, which is incorporated herein by reference. (Briefly, the origin of the Simian virus SV40 was isolated by digesting SV40 DNA with HindIII, and converting the HindIII ends to EcoRI ends by the addition of a converter (AGCTGAATTC)). This DNA was cut with PvuII, and RI linkers added. Following digestion with EcoRI, the 348 base-pair fragment spanning the origin was isolated by polyacrylamide gel electrophoresis and electroelution, and cloned in pBR322. Expression plasmid pHBs348-E was constructed by cloning the 1986 base-pair fragment resulting from EcoRI and BglII digestion of HBV (*Animal Virus Genetics*, (Ch. 5) Acad. Press, N.Y. (1980)) (which spans the gene encoding HBsAg) into the plasmid pML (Lusky *et at.*, *Nature*, 293: 79 (1981)) at the EcoRI and BamHI sites. (pML) is a derivative of pBR322 which has a deletion eliminating sequences which are inhibitory to plasmid replication in monkey cells). The resulting plasmid (pRI-Bgl) was then linearized with EcoRI, and the 348 base-pair fragment representing the SV40 origin region was introduced into the EcoRI site of pRI-Bgl. The origin fragment can insert in either orientation. Since this fragment encodes both the early and late SV40 promoters in addition to the origin of replication, HBV genes could be expressed under the control of either promoter depending on this orientation (pHBS348-E representing HBs expressed under control of the early promoter). pE342 is modified by partially digesting with Eco RI, filling in the cleaved site using Klenow DNA polymerase I, and ligating the plasmid back together, thus removing the Eco RI site preceding the SV40 origin in pE342. The resulting plasmid is designated pE342ΔR1.

[0049] The resultant sequence creates a stop codon (TAA) immediately after amino acid 300 of the gD gene. The transcription termination and polyadenylation sites for the truncated gD gene transcript are encoded by the 3' untrans-

lated region of the hepatitis B surface antigen gene (36).

[0050] The resulting vector was transfected (40) into a dhfr$^-$ (CHO cell line (39), and a suitable clone gG10.2 selected which produced the truncated gD protein and secreted it into the surrounding medium. The protein was extracted from the medium and the cells were tested for immunogenic activity. Figure 9 shows the results of immunoprecipitations of intra- and extra-cellular ^{35}S-methionine-labelled extracts.

[0051] Radioimmunoprecipitation of cell associated- and secreted-forms of gD. Cells were grown in Ham's F12 medium (Gibco) supplemented with 7 percent commercially dialyzed fetal bovine serum (Gibco), penicillin (100 u/ml), and streptomycin (100 u/ml). When the cultures were approximately 80 percent confluent, the medium was removed, the cells were washed twice with phosphate buffered saline (PBS), and labeling medium (Dulbecco's modified Eagle's medium containing one-tenth the normal concentration of methionine) was added to a final concentration of 0.05 ml/cm^2. ^{35}S-methionine (SJ.204, Amersham Int.) was added to a final concentration of 50—75 uCi/ml and the cells were grown for an additional 18—20 hr. After labeling, the medium was harvested and the cells were washed twice in PBS, and removed from the culture dish by treatment with PBS containing 0.02 percent EDTA. The cells were then solubilized in lysis buffer consisting of: PBS, 3 percent NP-40, 0.1 percent bovine serum albumin, 5×10^{-5} M phenylmethylsulfonyl fluoride, and 0.017 TIU/ml of apoprotinin and the resultant lysate was clarified by centrifugation of 12,000×g. For immunoprecipitation reactions cell lysates were diluted 3-fold with PBS and aliquots (typically 180 µl) were mixed with 2—5 µl of antisera and incubated at 4°C for 30 min. To immunoprecipitate the secreted form of gD, 500 µl of conditioned medium was incubated with 2 µl of antisera for 30 min at 4°C. Immune complexes were then adsorbed to fixed S. aureus cells by the method of Kessler (40a) and were precipitated by centrifugation at 12,000×g for 30 s. The S. aureus cells were then washed 3 times with wash buffer (PBS, 1 percent NP-40, 0.3 percent sodium dodecyl sulfate), and the immune complexes were eluted with 20 µl of polyacrylamide gel sample buffer (62.5 mM Tris-HCl buffer, pH 6.8 containing 10 percent glycerol, 5 percent 2-mercaptoethanol, 0.01 percent bromophenol blue) at 90°C for 3 min. After centrifugation for 30 s the supernatants were applied to 10 percent polyacrylamide slab gels according to the method of Laemmli (45). A, immunoprecipitation of full length membrane bound gD from the gD12 cell line. B, immunoprecipitation of the cell associated form of the truncated gD from lysates of two independently derived cell lines (1 and 2). C, immunoprecipitation of the truncated gD from the culture supernatants of the two cell lines shown in B. (-), indicates control rabbit antiserum (+), indicates rabbit anti-HSV-1 antiserum (Dako Corp.).

[0052] As can be seen, evident are an intracellular form of 35,000 Daltons and a secreted and apparently glycosylated extracellular gD protein.

Preparation of truncated gD used for immunization

[0053] gD10.2 cells were grown to confluence in polystyrene tissue culture roller bottles (Corning 25140) in F12 medium supplemented with 7 percent commercially dialyzed fetal calf serum, 50 µg/ml streptomycin and 0.3 µg glutamine. After reaching confluence the medium was removed and the cells were washed three times in the same medium lacking fetal calf serum and supplemented with 2 mg/ml Hepes buffer (serum free medium). The cells were then grown 3—4 days in serum free medium and the conditioned medium was then harvested and stored at -20°C. The medium was thawed at 37°C and centrifuged at 5000 rpm for 20 min. in a Sorvall GS-3 rotor. After centrifugation the pellet was discarded and the supernatant was concentrated in an ultrafiltration apparatus (Amicon) equipped with a YM-5 ultrafiltration membrane. The resultant preparation was concentrated approximately 150-fold relative to the starting material and contained approximately 8 mg of protein per liter. The preparation was then dialyzed extensively against phosphate buffered saline (PBS) and used for immunization without further purification.

Immunization of mice

[0054] Each 8-week old BALB/c mouse was immunized with 36 µg of protein contained in 200 µl of an emulsion consisting of 50 percent aqueous antigen and 50 percent complete Freund's adjuvant. Each mouse was immunized at multiple intradermal and subcutaneous site as follows: 25 µl in each rear footpad, 50 µl in the tail, and 100 µl distributed among 3—5 intradermal sites along the back. Four weeks after the primary immunization the mice were boosted with 36 µg of the protein as above with the exception that the emulsion was prepared with incomplete Freund's adjuvant. For the booster immunization each mouse received 200 µl of the antigen emulsion distributed as follows: 50 µl in the tail, 150 µl distributed among 5 intradermal sites along the back. 19 days after boosting approximately 500 µl of blood was collected from each mouse by tail bleeding. The sera obtained from this bleed was used for in vitro neutralization studies (see below). 37 days after boosting the mice were used for virus challenge studies. Control mice matched to the experimentals with regard to age, sex and strain were immunized with human serum albumin (15 µg per mouse) using the same protocol as with the experimentals.

In vitro neutralization

[0055] Sera from eleven mice immunized with concentrated gD10.2 culture supernatant were tested for the ability to neutralize HSV-1 in vitro. Serially diluted mouse serum (2-fold dilutions: 1:8 to 1:16384) were incubated with approximately 40 pfu of HSV-1 for 1 hr. at 37°C in Dulbecco's modified Eagle's medium (DMEM). After the serum incubation, each dilution was applied to approximately 40,000 Vero cells contained in each well of a 96 well tissue culture plate. After 3—4 days virus growth was determined by staining each well with 0.5 percent crystal violet. Wells in which virus growth occurred showed no staining. Neutralization titers were calculated by determining the highest serum dilution which prevented virus induced cell death. All of the sera tested (n=10) from mice immunized with gD10.2 supernatant material showed HSV-1 neutralization activity (range 1:16 to 1:512) and HSV-2 neutralization activity (range 1:8 to 1:16). Control mouse sera (n=8) failed to provide any neutralization. Serum obtained from a mouse immunized with HSV-1 gave a neutralizating titer of 1:32.

Virus challenge

[0056] Eleven mice immunized with concentrated gD10.2 supernatant and 13 control mice immunized with human serum albumin were challenged with 10,000,000 pfu of HSV-1 (MacIntyre strain) by intraperitoneal injection. 14 days after the injection of virus, none of the gD10.2 immunized mice showed any indication of viral infection. In the control group, 7 of the 13 mice were dead by day 14, 3 showed severe wasting and paralysis, and 3 looked healthy. Statistical analysis (two tailed Fisher exact test) revealed that the difference between the immunized and control groups was significant at the P=0.002 level. (see Table 1).

TABLE 1

Expt.	No. of mice	Antigen	HSV1 neut.[1]	HSV2 neut.[1]	HSV1[2,4] Paralyzed	Challenge	
						Dead	Alive
509C	11	gDtrunc[3]	1:16—1:512	1:8—1:16	0	0	11
509D	13	HSA	0	0	3	7	3

[1]. Mouse sera were tested for HSV1 and HSV2 neutralization activity 19 days after the second secreted gD booster vaccination. Serially diluted mouse sera (1:8—1:16384) were incubated with 40 forming units of HSV1 or HSV2 for 1 hour at 37°C. Each dilution was applied to 40,000 Vero cells contained in each well of 96 well microtitre wells. After 4 days, the cells were stained with 0.5 percent crystal violet. Neutralization titres were calculated by determining the highest serum dilution which prevented virus growth.

[2]. Mice were challenged by intraperitoneal injection of 1×10^7 plaque forming units of HSV1 (MacIntyre strain). Challenged mice were observed for a period of three weeks for HSV1 infection.

[3]. Each mouse was immunized with approximately 3 micrograms of secreted gD in a 50 percent aqueous, 50 percent Feunds adjuvant solution. Mice were immunized at multiple intradermal and subcutaneous sites. Four weeks after the primary immunization, mice were boosted. Mice wer challenged 19 days after the booster immunization. Control mice were immunized with an equivalent amount of human serum albumin (HSA).

[4]. Significant at p=0.002 level.

[0057] It was found that the truncated protein released into the medium from gD10.2 cells was effective to protect mice from a lethal infection from HSV-1.

Antigen preparation for HSV-2 virus challenge

[0058] Amplified gD10.2.2 cells, grown in the presence of 250 nM methotrexate, were seeded into roller culture bottles (850 cm^2) and were cultured in Ham's F12 medium (GIBCO) supplemented with 7 percent fetal bovine serum. After the cells reached confluence (approximately 3 days), the culture medium was removed, the cells were washed three times in phosphate buffered saline (PBS) to remove serum proteins, and new "serum free" culture medium was added. The serum free medium consisted of Ham's F12 medium containing 25 mM hepes buffer. The cells were then cultured for three days and the resultant conditioned medium was harvested and used for antigen preparation. Fresh serum-free medium was then added to the cells and the cycle of harvesting conditioned medium at three day intervals was repeated an additional one or two times until the cells died or no longer adhered to the culture surface. gD10.2.2 conditioned serum-free medium was then filtered and centrifuged at low speed to remove cellular debris, and the resultant material was then concentrated ten- to twenty- fold with an ultrafiltration device (YM-10 membrane, Amicon). The con-

centrated medium was then dialyzed overnight against PBS (3 changes of PBS, one liter per change). The resulting material was then assayed to determine the protein concentration and analyzed by polyacrylamide gel electrophoresis to determine protein composition and to estimate the purity of the preparation. The material prepared by this process was then used to immunize animals against HSV-2 infection as described below.

Immunization of mice against HSV-2 infection

[0059] Forty female BALB/c mice were obtained from the Charles River Laboratories (Boston, MA) and were immunized with the secreted gD protein (gDtrunc) or human serum albumin (HSA) at 12 weeks of age. For the primary immunization against the secreted gD protein, the antigen was adjusted to a concentration of approximately 70 ug per ml in phosphate buffered saline and was emulsified with an equal volume of complete Freund's adjuvant. Each mouse was then immunized with 200 µl of this emulsion distributed as follows: 50 µl subcutaneously at a site approximately 1 cm from the base of the tail, 25 µl subcutaneously in each rear footpad, and 100 µl distributed among 3—5 intradermal sites along the back. The mice were then boosted with the same antigen one month after the primary immunization. For the booster immunization the antigen was prepared by the same procedure as with the primary immunization with the exception that incomplete Freund's adjuvant replaced complete Freund's adjuvant. For the booster immunization, 200 µl of antigen emulsion was injected into each mouse and was distributed as follows: 50 µl in the tail, 25 µl subcutaneously in the loose skin above each thigh, and 100 ul distributed among 3—5 intradermal sites along the back. The control group of mice was immunized according to the same protocol as the experimental group of mice with the exception that human serum albumin replaced the secreted gD protein as the immunogen. Serum was collected from the mice 24 days after boosting for use in *in vitro* neutralization studies.

HSV-2 virus challenge

[0060] Both experimental (secreted gD injected) and control (HSA injected) groups of mice were challenged by an intraperitoneal injection of HSV-2 (MS strain) 31 days after the booster immunization. Each mouse received 2×10^5 pfu of virus in 100 µl of Dulbecco's modified Eagle's medium (DMEM) containing 10 percent fetal bovine serum. LD50 experiments revealed that this amount of virus represented 100—500 times the amount of virus required to kill 50 percent of a population of normal (uninjected) BALB/c mice. The virus injected mice were observed for a period of 3 weeks. All of the control mice (HSA injected) died within 9 days of the virus challenge. All of the mice vaccinated with the secreted gD protein survived the full three weeks and appeared normal (i.e., they did not exhibit wasting or paralysis).

TABLE 2

Expt.	No. of mice	Antigen	HSV1 neut.[1]	HSV2 neut.[1]	HSVI[2] Paralyzed	Challenge	
						Dead	Alive
579C	15	gDtrunc	1:1024—1:2048	1:512—1:1024	0	0	15
579D	25	HSA	0	0	0	25	0

[1] Mouse sera were tested for HSV1 and HSV2 neutralization activity 19 days after the second secreted gD booster vaccination. Serially diluted mouse sera (1:8—1:16384) were incubated with 40 forming units of HSV1 or HSV2 for 1 hour at 37°C. Each dilution was applied to 40,000 Vero cells contained in each well of 96 well microtitre wells. After 4 days, the cells were stained with 0.5 percent crystal violet. Neutralization titres were calculated by determining the highest serum dilution which prevented virus growth. Values indicated represent the average neutralization titers. [2] See text above for the details of the HSV-2 challenge.

[0061] Truncated glycoprotein D was purified from culture medium conditioned by the growth of the gD10.2 cell line previously described. The culture medium was concentrated by ultrafiltration and truncated gD was purified by immunoaffinity chromatography using an anti-gD-1 monoclonal antibody coupled to Sepharose 4B Truncated HSV-1 glycoprotein D (gD-1) was isolated from serum-free medium conditioned by the growth of gD10.2 cells as previously described. The cell-culture medium was concentrated by ultrafiltration using commercially available membranes (Amicon Corp.) and ammonium sulfate precipitation. gD-1 was then purified to near-homogeneity by immunoaffinity chromatography. The immunoaffinity column was prepared by coupling a monoclonal antibody, produced against HSV-1 to cross-linked sepharose (Pharmacia Fine Chemials) and eluted by a method similar to that described by Axen, *et al.* Nature *214*: 1302—1304 (1967). The major product in the medium of unfractionated culture medium conditioned by the gD10.2 cell line is the mature truncated form of gD-1 (~43—46 kd) and a precursor form of gD (~38—40 kd). On average the gD

protein represents 20—50 percent of the protein present in growth conditioned medium. Fractionation of this material by immuno-affinity chromatography resulted in a considerable enrichment of gD. It can be seen that the eluted material is free of all contaminating proteins detectable by silver staining. To determine whether purification of the protein by this protocol denatured the protein or disrupted the antigenic structure of the molecule, antigenicity studies with a variety of monoclonal antibodies were conducted. In these studies we found that all antibodies tested except those reactive with the carboxy-terminus reacted with the purified preparation. No difference in antibody binding behavior could be detected with the purified preparation relative to the material found in unfractionated culture supernatants.

[0062] To determine whether the purified gD-1 protein could be used effectively as the basis of a subunit vaccine to protect from genital infection by HSV-2, guinea pigs were vaccinated with gD-1 formulated in various adjuvants. In the first studies, purified gD was incorporated in complete Freund's adjuvant and injected in intramuscular and subcutaneous sites of female Hartley guinea pigs. Female Hartley guinea pigs 2 months of age and weighing approximately 250 g were purchased from Charles River Laboratories (Portage, MI). For studies using Freund's adjuvant, the primary immunization consisted of the injection of 30 ug of dG-1 emulsified in 50 percent complete Freund's adjuvant distributed as follows: 0.5 ml injected subcutaneously into the loose skin above the neck, and 0.5 ml injected intramuscularly into the thigh. After 31 days, the animals were boosted with the same amount of antigen incorporated in incomplete Feund's adjuvant. Control animals were injected according to the same protocol as the experimentals with the exception that adjuvant alone was injected. Experimental and control animals were challenged by intravaginal infection with HSV-2 19 days after boosting. In studies using alum-adjuvented gD-1, 30 ug of gD-1 incorporated in either alum-phosphate or alum-hydroxide (0.15 ml) was used for both the primary and the booster immunizations. Alum-adjuvented protein was injected by intramuscular injection into the hind legs. Animals were boosted 51 days after the primary immunization and challenged 27 days later with live virus. Each animal received one primary immunization containing 30 ug of purified protein incorporated in complete Feund's adjuvant, and one booster immunization (31 days later) of the same amount of antigen incorporated in incomplete Freund's adjuvant. All animals were challenged by intravaginal innoculation of HSV-2 19 days after the booster immunization. Table 3 indicates the results obtained from these studies. It can be seen that animals vaccinated with gD produced high levels of antibodies capable of preventing both HSV-1 and HSV-2 virus infection in an *in vitro* virus neutralization assay. We found that the sera from these animals neutralized HSV-1 slightly more effectively than HSV-2. This result is reasonable in view of the fact that the immunogen was derived from HSV-1, and that there are known to be type specific antigenic determinants on gD-1 (Eisenberg, R. J. *et al., J. Virol. 35*: 428 (1980); Pereira, L. *et al., Infect. and Immun. 29*: 724 (1980); Showalter, J. D. *et al., Infect. and Immun. 34*,: 684 (1981)). More impressive was the fact that all of the animals vaccinated with gD-1 were completed protected from the clinical manifestations of virus infection (i.e. redness, swelling, vesicle formation, ulceration, loss of urinary retention, and lethal encephalitis). Thirteen of the fourteen animals injected with adjuvant alone developed severe primary infections. There were numerous vesicles which typically coalesce to form acute ulcerations. In contrast, animals vaccinated with gD-1 gave no indication of virus infection. These results clearly indicated that gD-1 incorporated in complete Freund's adjuvant can provide effective protection from genital HSV-2 infection.

[0063] Because complete Freund's adjuvant is not acceptable for use in humans, we next wanted to determine whether gD-1 could provide protection from HSV-2 infection when formulated with an adjuvant suitable for human use. To this end, studies with alum precipitated protein complexes (J. S. Garvey *et al.*, in *Methods in Immunology* (1977) p. 185(17)) were initiated. Table 3 compares results obtained using gD-1 incorporated in the adjuvants alum-hydroxide, and alum-phosphate. In control studies, animals were vaccinated with adjuvant alone. It can be seen that both of the alum-based preparations elicited high levels of neutralizing antibodies against HSV-1, and that the neutralizing titers against HSV-1 were comparable to those elicited against gD-1 incorporated in complete Freund's adjuvant. However, the titers of antibody capable of neutralizing HSV-2 were significantly lower with gD-1 incorporated in either of the alum preparations than gD-1 incorporated in complete Freund's adjuvant. This result suggests that incorporation of gD-1 in alum results in the loss of one or more antigenic determinants common to HSV-1 and HSV-2, or that recognition of cross-reactive antigens is more effective when the protein is incorporated in Freund's adjuvant. These results also suggest that alum-hydroxide is a more effective adjuvant than alum-phosphate since the neutralizing titers to HSV-1 and HSV-2 are significantly higher with the former than with the latter.

[0064] Although the protection provided by the alum-adjuvant preparations was less effective than that obtained with the Freund's adjuvant preparations, it was none the less significant. While a number of animals showed signs of virus infection, the severity of the infections was considerably less than that obtained in adjuvant alone injected control animals. Thus the mean lesion score was 0.9 in animals vaccinated with the alum-phosphate vaccine formulation, and 0.7 in the alum-hydroxide based formulation as compared with a mean lesion score of 3.2 for the adjuvant injected control animals. According to the 4+ scale used for scoring lesion severity, the reduction from a mean lesion score of 3.2 to 0.7 corresponds to a reduction in clinical symptoms from several large vesicles (score of 3) to minor redness and swelling (score of 0.5). Interestingly, throughout these studies the average *in vitro* neutralization titer against HSV-2 correlated with the severity of clinical disease.

[0065] The results described above demonstrate that the clinical manifestations of primary HSV-2 genital infection

can be significantly reduced by vaccination with recombinant gD-1. The results obtained demonstrate that a single HSV-1 derived glycoprotein can provide complete protection from genital HSV-2 infection when administered in conjunction with a potent adjuvant.

TABLE 3

Protection of guinea pigs from genital HSV-2 infection by vaccination with recombinant HSV-1 glycoprotein D						
Immunogen	n	Average *In vitro* neutralization titer* (log 2)		Symptomatic**	Assymtomatic**	Mean+ lesion score
		HSV-1	HSV-2			
gD-1 in Freund's adjuvant	15	9.3±0.8	8.0±1.4	0	100	0
Freund's adjuvant alone	14	<3	<3	93	7	3.3±1.4
gD-1 in alum-phosphate	9	9.2±2.2	5.6±2.2	56	44	0.9±1.2
gD-1 in alum-hydroxide	9	10.9±1.2	6.7±1.5	43	67	0.6±1.1
alum-phosphate alone	10	<3	<3	90	10	3.2±1.2

In vitro neutralization studies were performed as previously described.

**Guinea pigs were judged to be symptomatic (exhibiting characteristics of virus infection) or assymptomatic (no signs of virus infection) according to the +4 scoring system described by Stanberry, L. R., *et al., J. Infect. Dis. 146*: 397 (1982). In summary: 0 no abnormalities; 1+ swelling and erythema; 2+, a few small vesicles; 3+, several large vesicles; 4+, several large ulcerated lesions.

+Mean lesion score was calculated by taking the arithmatic average of the maximum lesion score observed in the symptomatic animals in each group.

[0066] The advantages of using the truncated protein for diagnostic and vaccine applications is that, being secreted into the extracellular medium, it is contaminated with far fewer proteins than would be found in a whole-cell preparation.

[0067] It will be noted that the present invention uses a permanent cell line to produce the protein. Upon transfection the vector is incorporated into the genome of the cell line and can produce the protein without cell lysis. The cell line can thus be used for continuous production of the protein, especially in the truncated form which is secreted from the cell. For example, the cells expressing truncated protein can be continuously used in a perfusion system by constantly removing antigen-rich medium from the cells and replacing it with fresh medium.

[0068] The particular cell line used here was a CHO line deficient in dhfr production, transfected with a vector containing a dhfr marker. By exposing the cell line to methotrexate (Mtx) under suitable conditions (54) the dhfr production and hence the linked gD protein production can be amplified. Three cell lines derived by transfection of the truncated gD gene into dhfr$^-$ CHO cells were plated in parallel, labeled with ^{35}S-methionine, and immunoprecipitated as described in Figure 10. Lanes 1 and 2 indicate the amount of secreted gD immunoprecipitated from 500 µl of culture medium conditioned by two independently isolated cell lines before selection with methotrexate. Lane 3 indicates the amount of truncated gD immunoprecipitated from an equal volume of culture medium from a cell line (gD10.2.2) selected for growth in 250 mM methotrexate. Rabbit anti-HSV-1 antibodies (Dako Corp.) were used for the immunoprecipitations shown in lanes 1—3. Lane 4 represents a control immunoprecipitation of 500 µl of medium conditioned by the gD10.2.2 cell line with normal rabbit serum.

[0069] To quantitate the relative amounts of truncated gD secreted into the culture medium by cell lines before and after selection in methotrexate, a competitive ELISA assay was performed. gD12 cells expressing a membrane-bound form of gD were plated out and fixed with glutaraldehyde to the surface of 96 well microtiter plates as previously described. Conditioned medium from various cell lines known to produce the truncated gD was serially diluted across the microtiter plate and was incubated with a fixed quantity (2 µl) of rabbit anti-HSV-1 antibody (Dako Corp) for 1 hr at 20°C. Unbound antibody and soluble truncated gD-antibody complexes were removed by washing each well 3 times with PBS. Horseradish peroxidase coupled to goat anti-rabbit IgG was then reacted with the fixed cells for 1 hr at 20°C and unbound antibody was removed by washing 3 times with PBS. The colorometric substrate, OPD (o-phenylene diamine), was then added to each well and allowed to react with the bound horseradish peroxidase-antibody complexes

for 15 min. The reaction was terminated by the addition of sulfuric acid to a final concentration of 0.25 N. The absorbance of the OPD in each well was determined with the use of an automated microtiter plate scanner (Titertek multiskan) and dilution curves were plotted. The binding of anti-HSV-1 antibodies to the parental CHO cell line was used to measure the extent of nonspecific binding at each dilution. The amount of truncated gD in each culture supernatant was inversely proportional to the amount of absorbance in each well. Open circle, binding of anti-HSV-1 antibodies to gD12 cells in the presence of medium conditioned by cells secreting truncated gD before amplification with methotrexate. Closed circle, binding of anti-HSV-1 antibodies to gD12 cells in the presence of medium from gD10.2.2 cells selected for growth in 250 nM methotrexate. Open square, binding of anti-HSV-1 antibodies to gD12 cells in the presence of 100-fold concentrated medium from unamplified cells secreting truncated gD. This procedure was carried out on the gD10.2 cell line to produce an amplified cell line gD10.2.2 which was capable of growth in 250 nM Mtx and which secreted approximately 20-fold more truncated gD into the culture medium than the parental gD10.2 cell line (see Figures 10 and 11).

[0070] The dhfr marker/amplification system can be used with other cells which are able to acquire and stably incorporate foreign DNA.

[0071] The success of this invention in demonstrating that a truncated form of a membrane bound protein, lacking that part of the hydrophobic-hydrophilic carboxy-terminal region responsible for binding it to the membrane, can yet be immunogenic indicates that similar results can be expected with other immunogenic membrane bound proteins, thus providing an improved source of vaccine against viruses, parasites and other pathogenic organisms.

[0072] In the foregoing example, the DNA of gD protein was truncated at residue 300 because there was a convenient restriction site there. This had the result that the carboxy-terminal hydrophobic/hydrophilic region was completely removed, as can be seen from the hydropathy plot of Figure 2; indeed an additional preceding region was removed from residue 301 to 332 without, apparently, destroying the immunogenic character of the protein. It would seem to follow, therefore, that with this protein, and probably with other immunogenic membrane bound proteins, the extent of truncation could be considerably less if desired, so long as it has the effect of removing the membrane binding character so that the protein is secreted into the surrounding medium.

[0073] Previous sequence comparison of the gD genes of HSV-1 and HSV-2 (58) demonstrated that the amino-terminal signal sequence (63) and the carboxy-terminal transmembrane domain (64) were able to tolerate a large number of mutations as long as the substituted amino acids were hydrophobic. The gC and gF sequence comparison demonstrates a similar finding in the carboxy-terminal, putative transmembrane domain (64) from residues 476—496 of gC and 443—463 of gF. The large number of heterologous hydrophobic substitutions in this region suggests that, as in gD, any amino acid which is lipid-soluble can be tolerated in this region. In contrast to gD, however, the amino-terminal signal sequences of gC and gF are highly homologous in the first 19 residues. Thus, either this region has an important conserved function other than direction of the glycoproteins into the rough endoplasmic reticulum (5), or there may be an overlapping gene or other functional sequence in this region of the genome which must be conserved (66).

[0074] It is believed that the cloned gC-2 glycoproteins may be expressed and formed into a vaccine in a manner analogous to that set forth in Example 1.

[0075] It is further believed that a vaccine which includes a mixture of such recombinant gC and gD glycoproteins would be significantly more effective as a vaccine against HSV-1 and HSV-2 than one based upon either glycoprotein alone.

[0076] The references grouped in the following bibliography and respectively cited parenthetically by letter and number in the foregoing text, are hereby incorporated by reference.

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Claims

1. A process which comprises producing a truncated, membrane-free derivative of a glycoprotein D of Herpes simplex virus type 1 or type 2, said derivative being devoid of membrane-binding domain whereby the derivative polypeptide is free of said membrane, and having exposed antigenic determinants that raise neutralizing antibodies and provide protection in an immunised subject against in vivo challenge by Herpes simplex virus type 1 and/or type 2, said method comprising expressing DNA encoding said derivative in a stable eukaryotic cell line transfected with said DNA.

2. A process according to Claim 1 which further includes collecting the truncated polypeptide as a secretion product.

3. A process according to Claim 1 or Claim 2 which includes the preliminary steps of preparing DNA encoding said membrane-bound polypeptide but lacking coding for membrane-binding domain, incorporating the DNA into an expression vector and transfecting said eukaryotic host cell with said vector.

4. A process according to any one of Claims 1, 2 and 3 wherein the transfected host cell is a mammalian cell line.

5. A process according to Claim 4 wherein the cell line is deficient in the production of dhfr and the vector contains a dhfr selectable marker.

6. A process according to any one of the preceding claims, wherein the truncated derivative comprises the N-terminal region of gD polypeptide up to about amino acid residue 300.

7. A process according to any one of the preceding claims which further comprises formulating a vaccine with the polypeptide.

8. A vaccine comprising a glycosylated, truncated, membrane-free derivative of a membrane-bound viral polypeptide, said derivative being the product of expression in, and secretion from, a eukaryotic host cell of recombinant DNA encoding said viral polypeptide but lacking the membrane-binding domain whereby the derivative polypeptide is an immunogenic recombinant secretion product free of said membrane and having exposed antigenic determinants that raise neutralizing antibodies and provide protection in an immunised subject against in vivo challenge by a viral pathogen, the truncated polypeptide being a derivative of a glycoprotein D of a herpes simplex virus type 1 or 2, and the pathogen is herpes simplex virus type 1 and/or type 2.

9. A vaccine according to Claim 8, wherein the truncated derivative comprises the N-terminal region of gD polypeptide up to about amino acid residue 300.

Patentansprüche

1. Verfahren umfassend die Herstellung eines verkürzten membranfreien Derivats eines Glykoproteins D von Herpes simplex Virus vom Typ 1 oder Typ 2, wobei dem Derivat die membranbindende Domäne fehlt, wodurch das Derivat-Polypeptid frei von der Membran ist, und es exponierte Antigen-Determinanten besitzt, die neutralisierende Antikörper erzeugen und bei einem immunisierten Individuum Schutz gegen in vivo-Infektion mit Herpes simplex Virus

vom Typ 1 und/oder Typ 2 bieten, wobei das Verfahren das Exprimieren von für das Derivat kodierender DNA in einer mit der DNA transfizierten, stabilen, eukaryotischen Zell-Linie umfaßt.

2. Verfahren nach Anspruch 1, welches weiters das Sammeln des verkürzten Polypeptids als Ausscheidungsprodukt umfaßt.

3. Verfahren nach Anspruch 1 oder 2, welches die vorausgehenden Schritte der Herstellung von DNA, die für das membrangebundene Polypeptid kodiert, der aber die Kodierung für die membranbindende Domäne fehlt, des Einbringens der DNA in einen Expressionsvektor und des Transfizierens der eukaryotischen Wirtszelle mit dem Vektor umfaßt.

4. Verfahren nach einem der Ansprüche 1, 2 und 3, worin die transfizierte Wirtszelle eine Säugetier-Zell-Linie ist.

5. Verfahren nach Anspruch 4, worin die Zell-Linie defizient in der Produktion von dhfr ist und der Vektor eine dhfr-selektierbare Markierung enthält.

6. Verfahren nach einem der vorangegangenen Ansprüche, worin das verkürzte Derivat einen N-terminalen Bereich von gD-Polypeptid bis zu etwa Aminosäurerest 300 umfaßt.

7. Verfahren nach einem der vorangegangenen Ansprüche, welches weiters das Formulieren eines Impfstoffs mit dem Polypeptid umfaßt.

8. Impfstoff, umfassend ein glykosyliertes, verkürztes, membranfreies Derivat eines membrangebundenen viralen Polypeptids, wobei das Derivat das Produkt der Expression von rekombinanter DNA in und Ausscheidung aus einer eukaryotischen Wirtszelle ist, die für das virale Polypeptid kodiert, der aber die membranbindende Domäne fehlt, wodurch das Derivat-Polypeptid ein immunogenes, rekombinantes Ausscheidungsprodukt ist, das frei von der Membran ist und exponierte Antigen-Determinanten besitzt, die neutralisierende Antikörper erzeugen und bei einem immunisierten Individuum Schutz gegen eine in vivo-Infektion mit einem viralen Pathogen bietet, wobei das verkürzte Polypeptid ein Derivat eines Glykoproteins D von Herpes simplex Virus vom Typ 1 oder Typ 2 und das Pathogen Herpes simplex Virus Typ 1 und/oder Typ 2 ist.

9. Impfstoff nach Anspruch 8, worin das verkürzte Derivat den N-terminalen Bereich des gD-Polypeptids bis zu etwa Aminosäurerest 300 umfaßt.

Revendications

1. Procédé qui comprend la production d'un dérivé tronqué, sans membrane, d'une glycoprotéine D de virus d'herpès simplex de type 1 ou de type 2, ledit dérivé étant dépourvu du domaine de liaison à la membrane, le polypeptide dérivé étant ainsi dégagé de ladite membrane, et ayant des déterminants antigéniques exposés qui engendrent des anticorps neutralisants et confèrent une protection chez un sujet immunisé contre une épreuve in vivo par le virus d'herpès simplex de type 1 et/ou de type 2, ladite méthode comprenant l'expression d'un ADN codant pour ledit dérivé dans une lignée de cellules eucaryotiques stables transfectée avec ledit ADN.

2. Procédé suivant la revendication 1, qui comprend en outre l'étape consistant à recueillir le polypeptide tronqué sous forme d'un produit de sécrétion.

3. Procédé suivant la revendication 1 ou la revendication 2, qui comprend les étapes préliminaires de préparation de l'ADN codant pour ledit polypeptide lié à la membrane mais non codant pour le domaine de liaison à la membrane, d'incorporation de l'ADN à un vecteur d'expression, et de transfection de ladite cellule hôte eucaryotique avec ledit vecteur.

4. Procédé suivant l'une quelconque des revendications 1, 2 et 3, dans lequel la cellule hôte transfectée est une lignée cellulaire de mammifère.

5. Procédé suivant la revendication 4, dans lequel la lignée cellulaire est déficiente en production de dhfr et le vecteur contient un marqueur sélectionnable dhfr.

6. Procédé suivant l'une quelconque des revendications précédentes, dans lequel le dérivé tronqué comprend la

région N-terminale du polypeptide gD jusqu'à approximativement le résidu d'aminoacide 300.

7. Procédé suivant l'une quelconque des revendications précédentes, qui comprend en outre la formulation d'un vaccin avec le polypeptide.

8. Vaccin comprenant un dérivé tronqué glycosylé, sans membrane, d'un polypeptide viral lié à la membrane, ledit dérivé consistant en le produit de l'expression dans, et de la sécrétion par, une cellule hôte eucaryotique d'un ADN recombinant codant pour ledit polypeptide viral mais étant dépourvu du domaine de liaison à la membrane, le polypeptide dérivé étant ainsi un produit de sécrétion recombinant immunogène dégagé de ladite membrane et ayant des déterminants antigéniques exposés qui engendrent un anticorps neutralisant et confèrent une protection chez un sujet immunisé contre une épreuve in vivo par un agent pathogène viral, le polypeptide tronqué étant un dérivé d'une glycoprotéine D d'un virus d'herpès simplex de type 1 ou 2, et l'agent pathogène consistant en virus d'herpès simplex de type 1 et/ou de type 2.

9. Vaccin suivant la revendication 8, dans lequel le dérivé tronqué comprend la région N-terminale du polypeptide gD jusqu'à approximativement le résidu d'aminoacide 300.

Fig. 1A(Part 1)

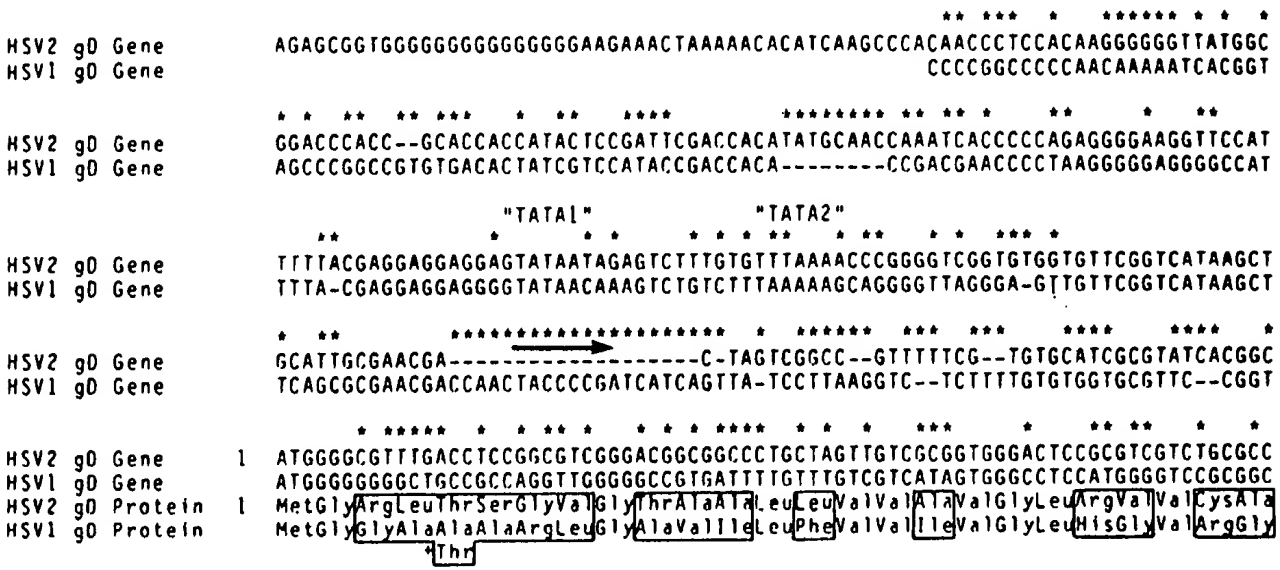

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Fig. 1A (Part 2)

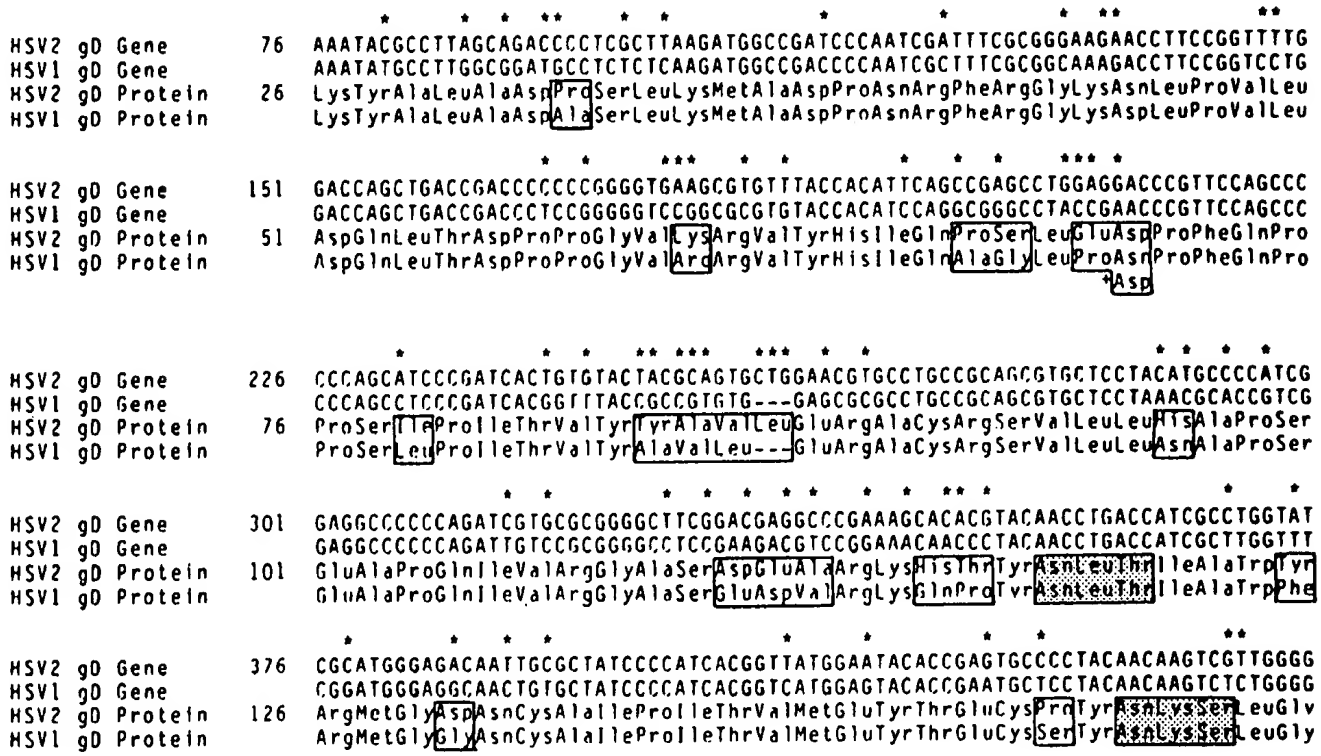

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Fig.1A(Part 3)

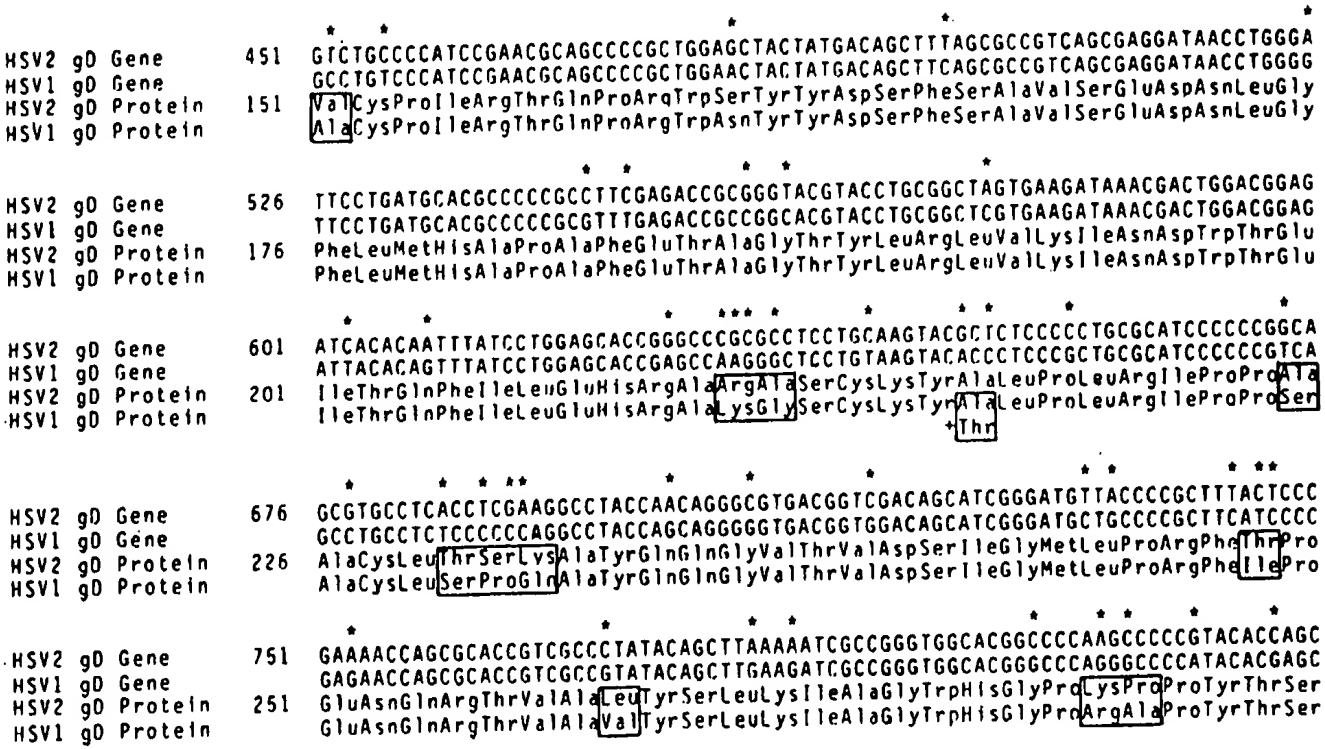

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Fig.1B(Part 1)

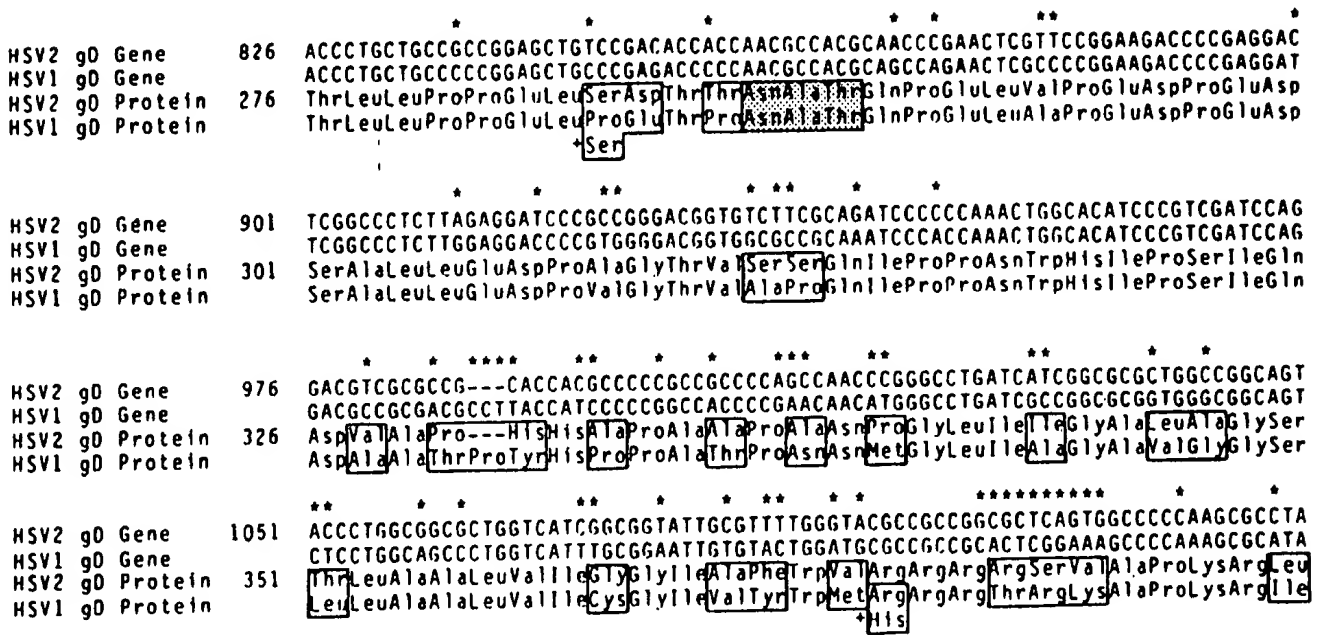
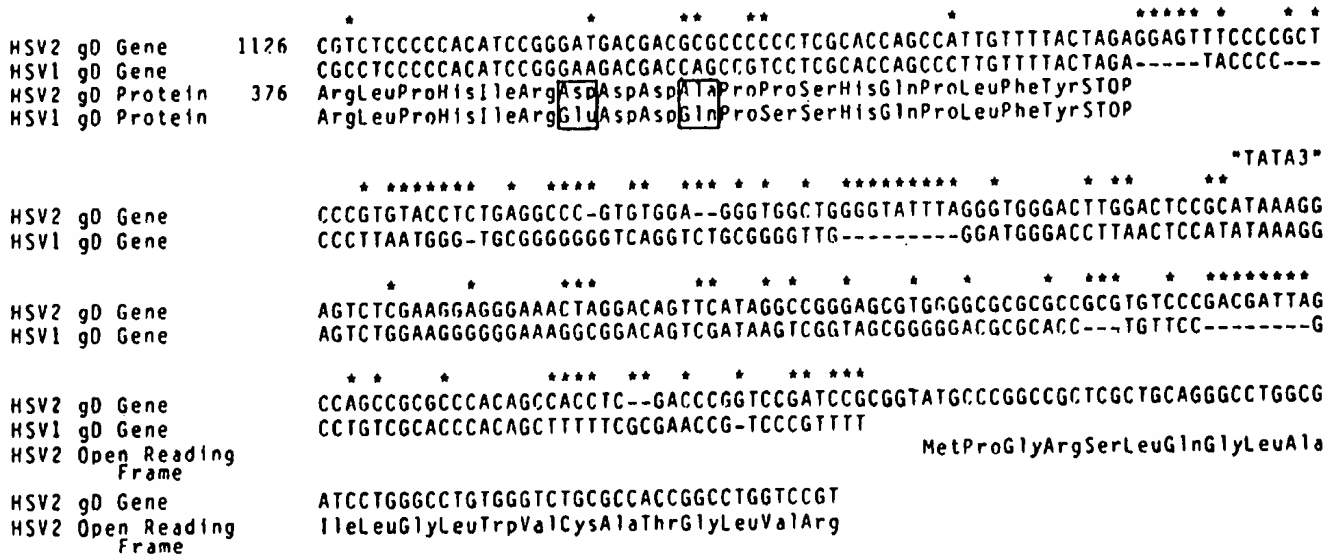

HSV2 gD Gene 826 ACCCTGCTGCCGCCGGAGCTGTCCGACACCACCAACGCCACGCAACCCGAACTCGTTCCGGAAGACCCCGAGGAC
HSV1 gD Gene ACCCTGCTGCCCCCGGAGCTGCCCGAGACCCCCAACGCCACGCAGCCAGAACTCGCCCCGGAAGACCCCGAGGAT
HSV2 gD Protein 276 ThrLeuLeuProProGluLeuSerAspThrThrAsnAlaThrGlnProGluLeuValProGluAspProGluAsp
HSV1 gD Protein ThrLeuLeuProProGluLeuProGlyThrProAsnAlaThrGlnProGluLeuAlaProGluAspProGluAsp
+Ser
HSV2 gD Gene 901 TCGGCCCTCTTAGAGGATCCCGCCGGGACGGTGTCTTCGCAGATCCCCCCAAACTGGCACATCCCGTCGATCCAG
HSV1 gD Gene TCGGCCCTCTTGGAGGACCCCGTGGGGACGGTGGCGCCGCAAATCCCACCAAACTGGCACATCCCGTCGATCCAG
HSV2 gD Protein 301 SerAlaLeuLeuGluAspProAlaGlyThrValSerSerGlnIleProProAsnTrpHisIleProSerIleGln
HSV1 gD Protein SerAlaLeuLeuGluAspProValGlyThrValAlaProGlnIleProProAsnTrpHisIleProSerIleGln
HSV2 gD Gene 976 GACGTCGCGCCG---CACCACGCCCCCGCCGCCCCAGCCAACCCGGGCCTGATCATCGGCGCGCTGGCCGGCAGT
HSV1 gD Gene GACGCCGCGACGCCTTACCATCCCCCGGCCACCCCGAACAACATGGGCCTGATCGCCGGCGCGGTGGGCGGCAGT
HSV2 gD Protein 326 AspValAlaPro---HisHisAlaProAlaAlaProAlaAsnProGlyLeuIleIleGlyAlaLeuAlaGlySer
HSV1 gD Protein AspAlaAlaThrProTyrHisProProAlaThrProAsnAsnMetGlyLeuIleAlaGlyAlaValGlyGlySer
HSV2 gD Gene 1051 ACCCTGGCGGCGCTGGTCATCGGCGGTATTGCGTTTTGGGTACGCCGCCGGCGCTCAGTGGCCCCAAGCGCCTA
HSV1 gD Gene CTCCTGGCAGCCCTGGTCATTTGCGGAATTGTGTACTGGATGCGCCGCCGCACTCGGAAAGCCCCAAAGCGCATA
HSV2 gD Protein 351 ThrLeuAlaAlaLeuValIleGlyGlyIleAlaPheTrpValArgArgArgArgSerValAlaProLysArgLeu
HSV1 gD Protein LeuLeuAlaAlaLeuValIleCysGlyIleValTyrTrpMetArgArgArgThrArgLysAlaProLysArgIle
+His

Fig. 1B(Part 2)



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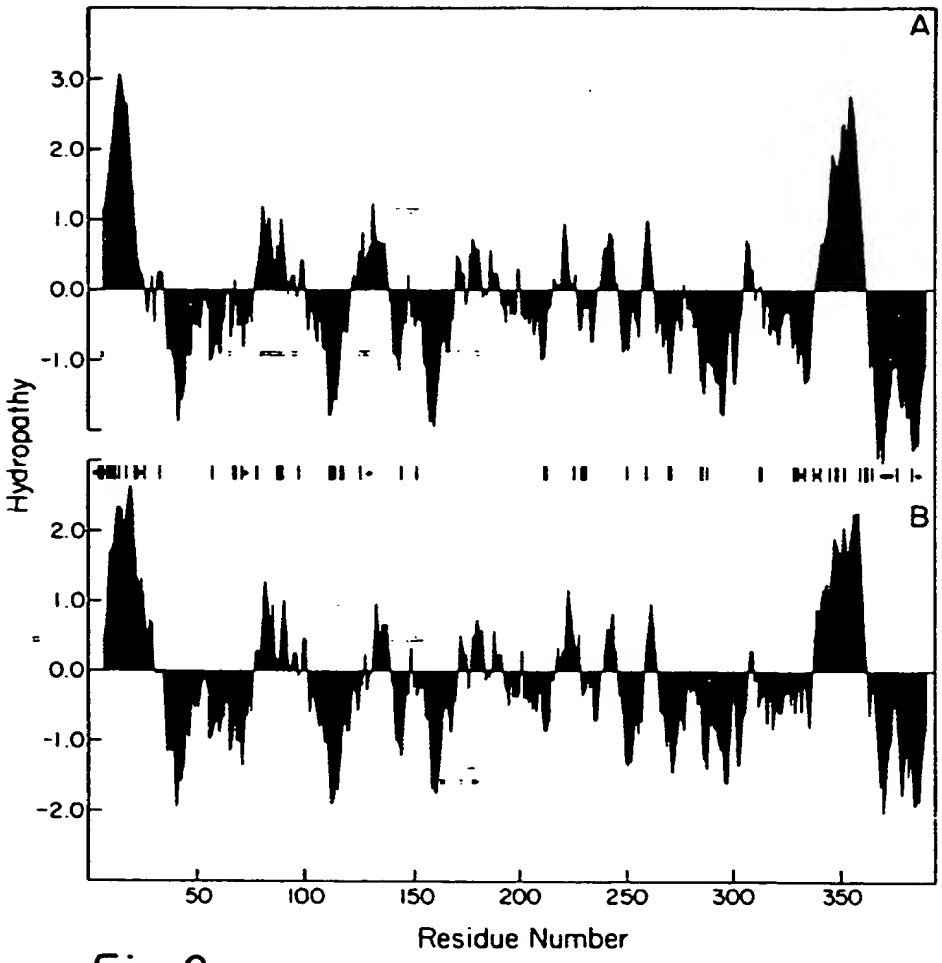

A
B
3.0
2.0
1.0
0.0
-1.0
-2.0
Hydropathy
50
100
150
200
250
300
350
Residue Number

Fig. 2.

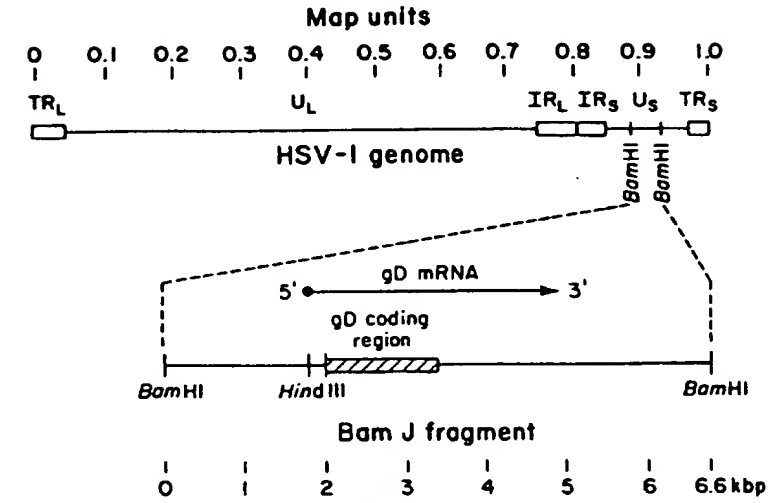


Fig. 3.

1. Digest with *Hin*dIII and *Bam*HI
2. Isolate 4.6kb gD encoding fragment
3. Ligate into *Hin*dIII – *Bam*HI cleaved DHFR vector

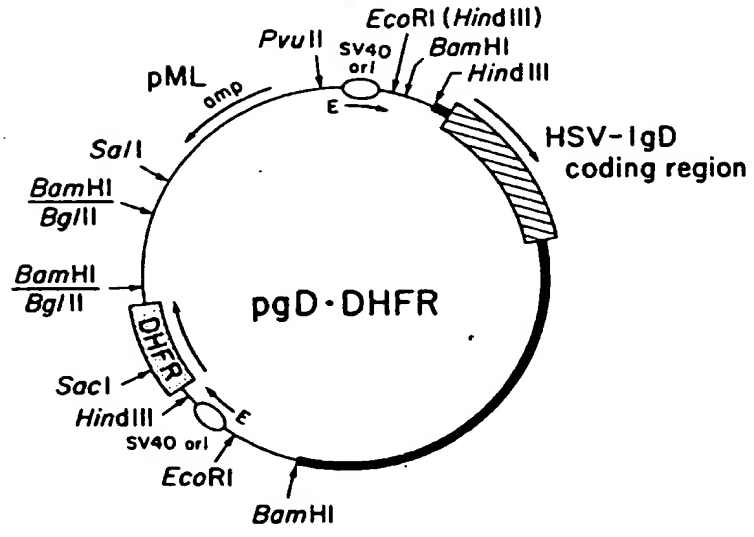


Fig.4.

A

B

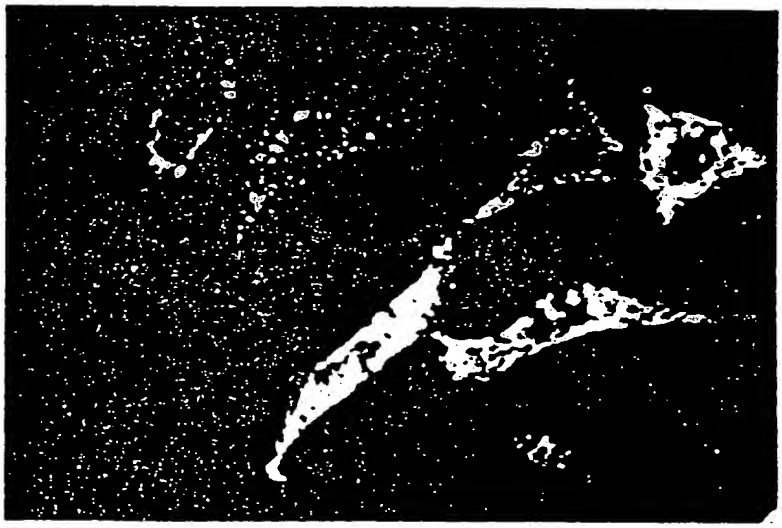


Fig.5.

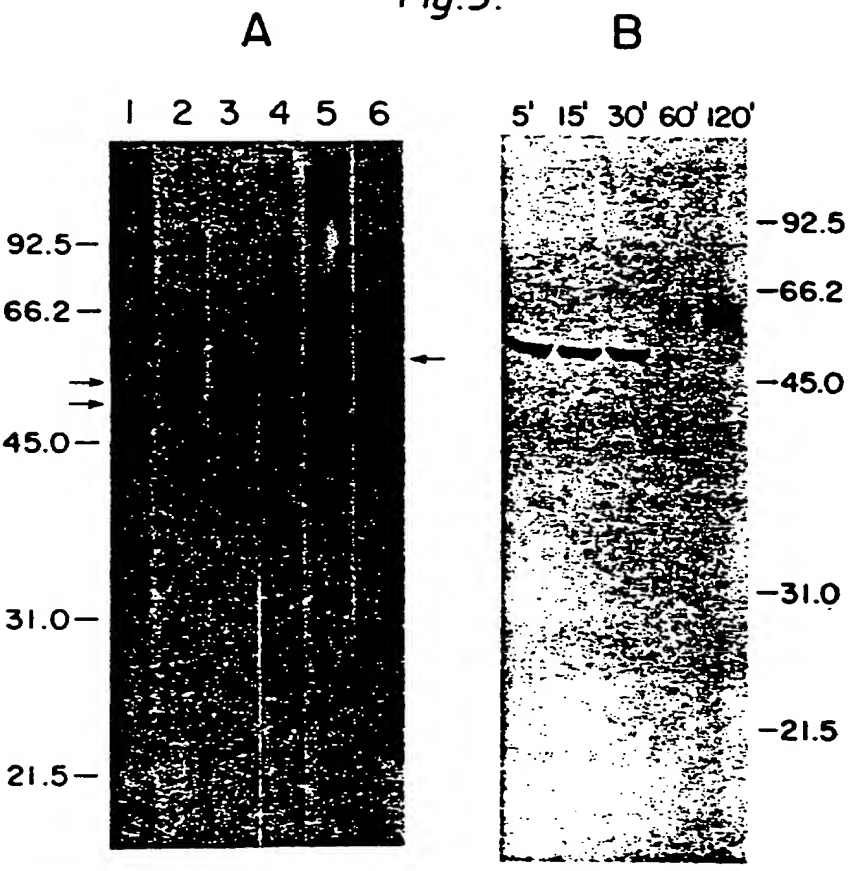
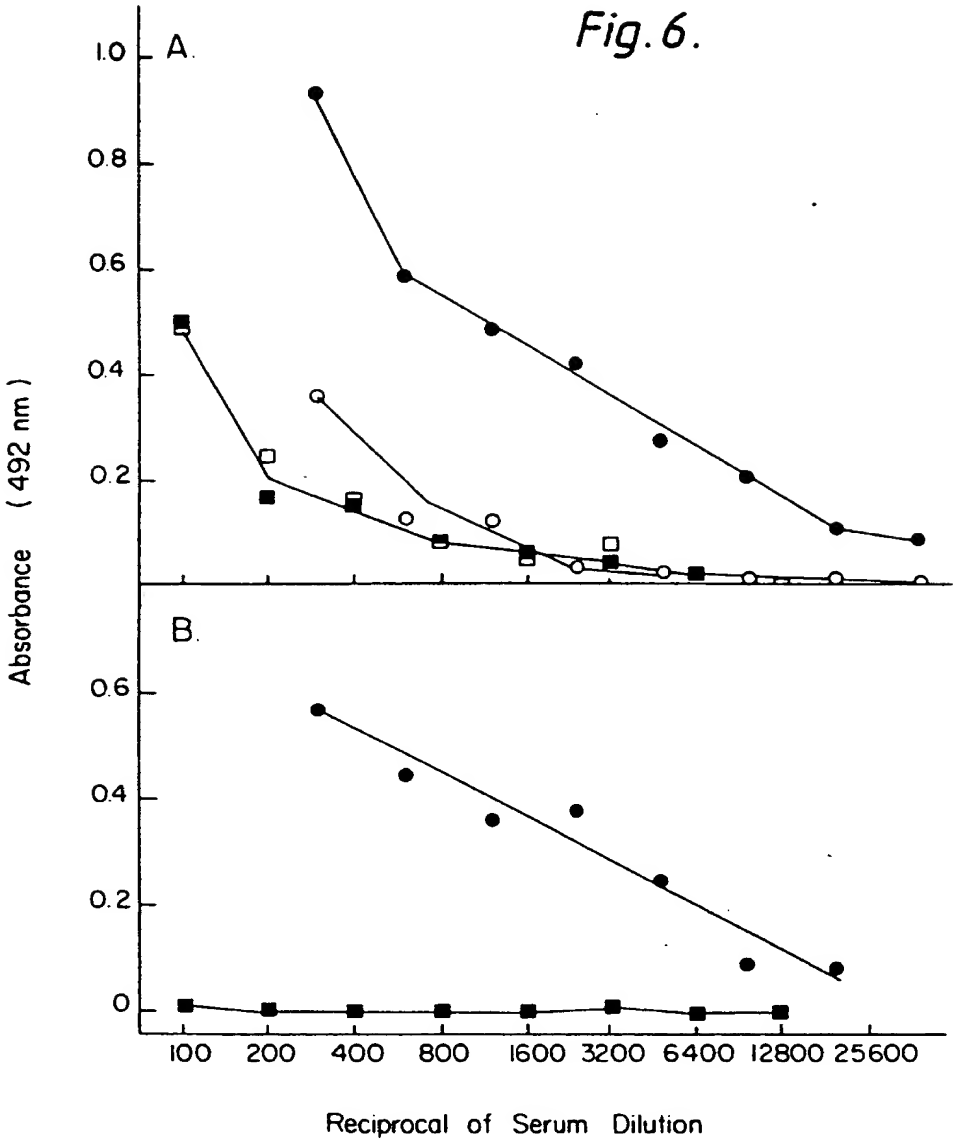


Fig. 6.


A.
B.
1.0
0.8
0.6
0.4
0.2
0
Absorbance (492 nm)
100
200
400
800
1600
3200
6400
12800
25600
Reciprocal of Serum Dilution

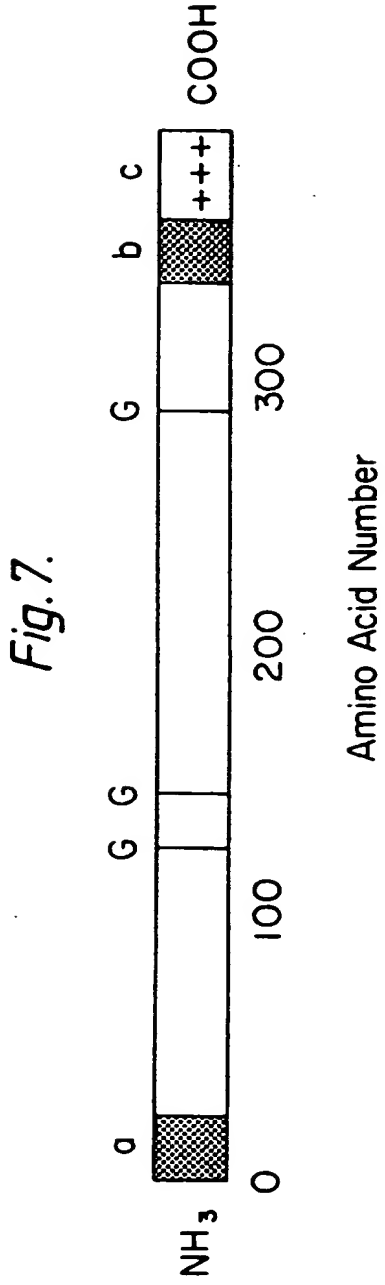


Fig. 7.

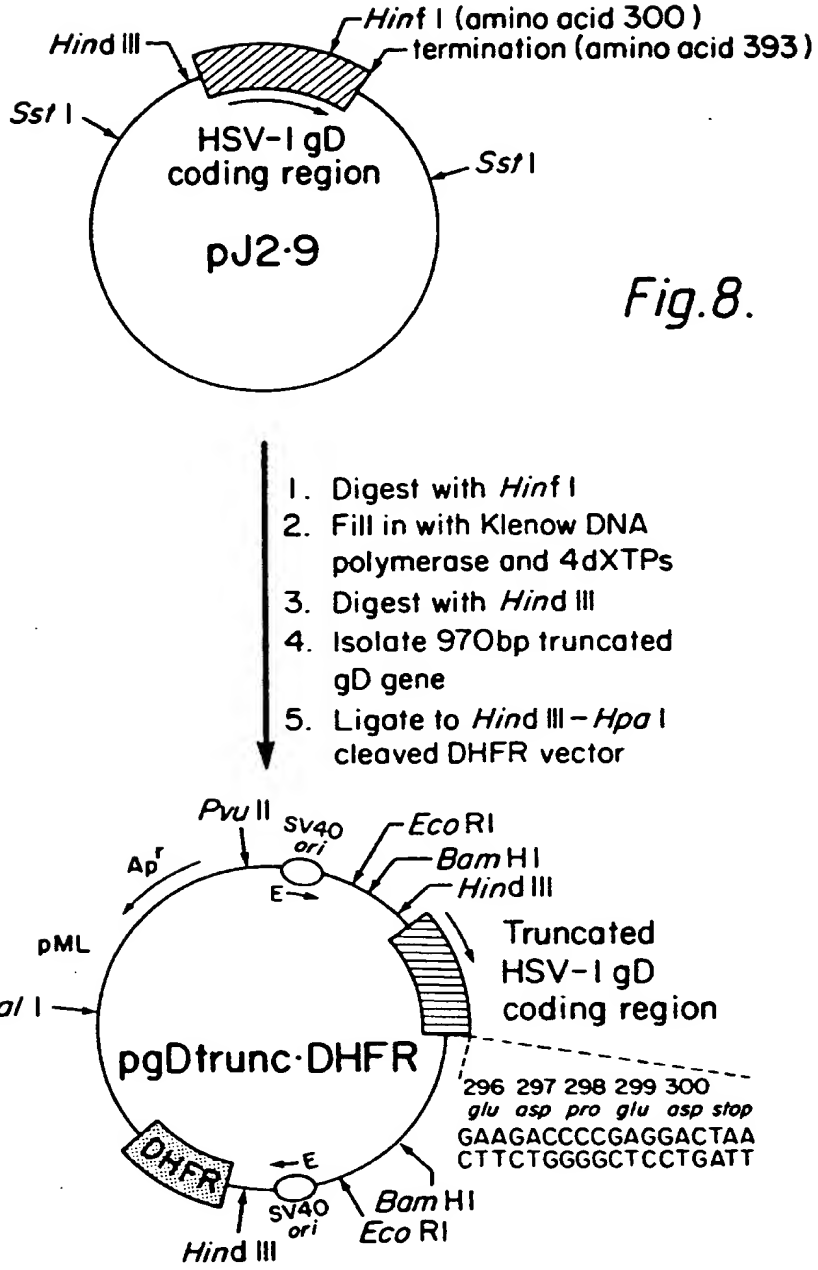

Hinf I (amino acid 300)
termination (amino acid 393)
Hind III
Sst I
HSV-1 gD
coding region
Sst I
pJ2·9
1. Digest with Hinf I
2. Fill in with Klenow DNA polymerase and 4dXTPs
3. Digest with Hind III
4. Isolate 970bp truncated gD gene
5. Ligate to Hind III – Hpa I cleaved DHFR vector
Pvu II
SV40 ori
Eco RI
Bam HI
Hind III
Ap^r
E
pML
Truncated
HSV-1 gD
coding region
Sal I
pgDtrunc·DHFR
296 297 298 299 300
glu asp pro glu asp stop
GAAGACCCCGAGGACTAA
CTTCTGGGGCTCCTGATT
DHFR
E
SV40 ori
Bam HI
Eco RI
Hind III

Fig.8.

Fig. 9.

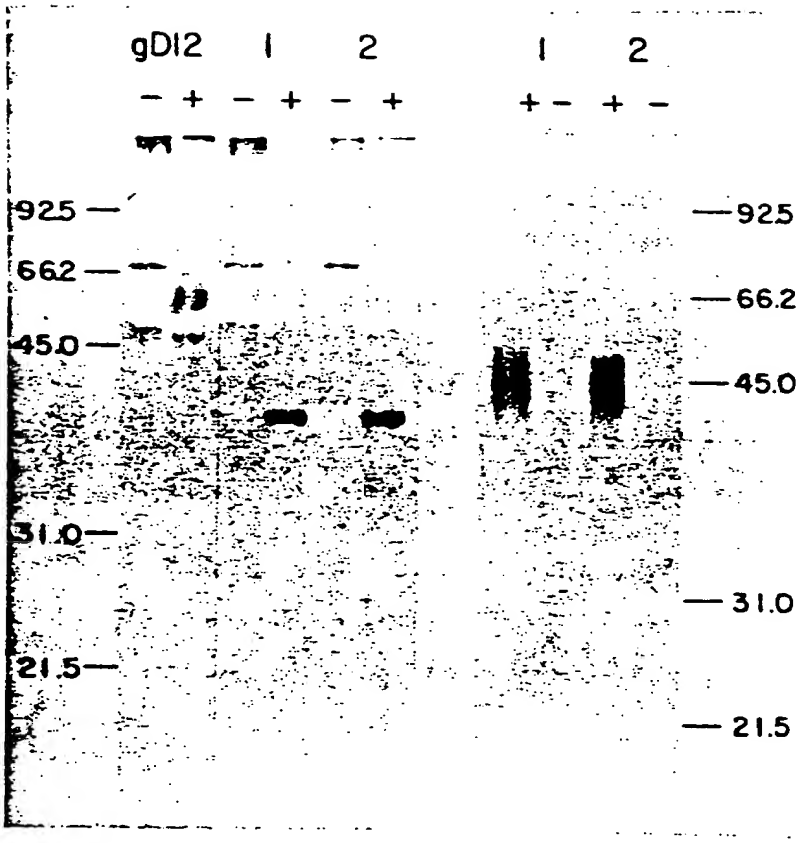


Fig.10.

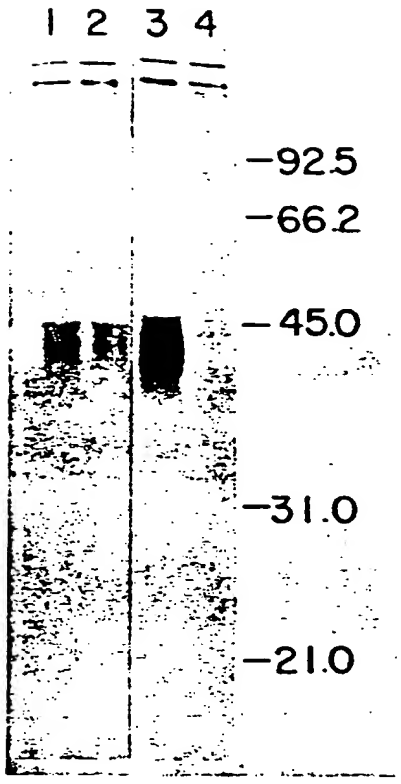


Fig. 11.